PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 06 November 2000 (06.11.00)	
International application No. PCT/EP00/01877	**Applicant's or agent's file reference** 10630
International filing date (day/month/year) 06 March 2000 (06.03.00)	**Priority date** (day/month/year) 09 March 1999 (09.03.99)
Applicant FLOHÉ, Leopold et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

09 October 2000 (09.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10630		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/01877	06/03/2000	09/03/1999
International Patent Classification (IPC) or national classification and IPC G01N33/573		
Applicant FLOHE, Leopold et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09/10/2000	08.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thumb, W Telephone No. +49 89 2399 7350

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/01877

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-8 as originally filed

Drawings, sheets:

1/4-4/4 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-8
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item II

Priority

The document Ursini et al. 'Dual function of the selenoprotein PHGPx during sperm maturation' Science, 285, p. 1393-1395, indicated in the search report as an intermediate document is not to be regarded as state of the art according to Rule 64.1 PCT, as the date of priority claimed can be allowed for the relevant parts of the present application.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: MAIORINO M. ET AL.: 'Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation' FASEB J., vol. 12, 1998, pages 1359-1370

   D2: MAIORINO M. ET AL.: 'Phospholipid hydroperoxide glutathione peroxidase' METHODS ENZYMOL., vol. 186, 1990, pages 448-457

2. Novelty

   Claims 1-7 and 8 are novel within the meaning of Article 33(2) PCT, since a method for determination of latent phospholipid hydroperoxide glutathione peroxidase (PHGPx) comprising the use of chaotropic agents for solubilizing a sperm sample and reactivation of the enzyme by using high concentrations of thiols, and the use of said method in predicting the fertilization potential of spermatozoa, respectively, do not form part of the state of the art known to the examining authority.

3. Inventive step

3.1 Document D1, which is considered to represent the most relevant state of the art, discloses a method for the determination of PHGPx in a sperm sample. A testis homogenate from rats is obtained, wherein after decapsulation, testes are diluted in buffer containing 1% Triton X-100 and 5 mM beta-mercaptoethanol. Cells are lysed using a freeze-thawing technique and, after removal of cellular debris, the supernatant is assayed for enzyme activity, following an additional purification step using size exclusion chromatography (page 1360, column 2, lines 35-50). It is shown that PHGPx is the main selenoperoxidase in rat testis and correlates with sexual maturation.

3.2 The subject-matter of claim 1 differs from the teaching of D1 in that the method of lysing spermatozoa comprises the use of chaotropic agents and the addition of high concentrations of thiols.
The underlying objective problem may therefore be seen in providing a method for reactivating PHGPx, which has lost its enzyme activity, in a sample preparation prior to assaying said activity.
In document D2, it is noted that a substantial activity of PHGPx is present in membranes of subcellular organelles, which can be partially recovered by high ionic strength extraction (page 449, lines 6-9). Two alternatives for preparation of PHGPx from tissue are disclosed (page 455, lines 13-36).
However, no indication can be found in the state of the art that would prompt the skilled person to denature PHGPx using chaotropic agents and reactivate enzymatic activity by adding a high concentration of thiols prior to a functional assay in order to solve the above stated problem.
Claim 1 is therefore considered as being inventive within the meaning of Article 33(3) PCT.

3.3 Dependent claims 2-7 refer to further specific embodiments of the method of claim 1 and consequently also meet the requirements of Article 33(3) PCT.

3.4 Claim 8 also complies with the provisions of Article 33(3) PCT since the use of the novel and inventive method of claims 1-7 in predicting the fertilizing potential of spermatozoa is neither disclosed nor rendered obvious in the prior art.

Re Item VII

Certain defects in the international application

The term IPG-phor used in the description, page 12, line 26, appears to be a registered trade mark and should be identified as such.

Re Item VIII

Certain observations on the international application

1. The relative term "high concentration" used in claim 1 has no well-recognised meaning and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT, see also PCT Guidelines III-4.5).

2. Claim 1 is not supported by the description as required by Article 6 PCT, as its scope is broader than justified by the description, the reason being as follows: Claim 1 discloses the addition of chaotropic agents as well as high concentrations of reducing agents to a sample, and the determination of enzymatic activity of PHGPx. However, based on the teaching of the description (e.g. page 7, lines 13-14; page 17, lines 8-12), removal of low molecular weight compounds appears to be an essential step before assessing the enzymatic activity of the protein. Since it appears unlikely that a protein exhibits its function in a denatured state (i.e. in a solution containing chaotropic agents), removal of said low molecular weight compounds should be included in the wording of claim 1 to meet the requirements of Article 6 PCT.

3. The vague and imprecise statement in the description on page 3, lines 13-21, implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in a lack of clarity of the claims (Article 6 PCT) when used to interpret them (see also PCT-Guidelines, III-4.3a.).

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
10630		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 01877	06/03/2000	09/03/1999
Applicant		
FLOHE, Leopold		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No 1

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure

   [ ] because this figure better characterizes the invention

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01877

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/573 G01N33/561 C12Q1/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROVERI A. ET AL.: "Enzymatic and immunological measurements of soluble and membrane bound PHGPx" METHODS ENZYMOL., vol. 233, 1994, pages 202-212, XP000921475 cited in the application page 204, paragraph 5 -page 210, paragraph 1 ---	1-8
X	MAIORINO M. ET AL.: "Phospholipid hydroperoxide glutathione peroxidase" METHODS ENZYMOL., vol. 186, 1990, pages 448-457, XP000921458 page 452, paragraph 3 -page 455, paragraph 5 --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2000	25/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

3

Form PCT ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01877

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 13225 A (BETH ISRAEL HOSPITAL) 9 May 1996 (1996-05-09) claims 12-19	1-8
X	MAIORINO M. ET AL.: "Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation" FASEB J., vol. 12, 1998, pages 1359-1370, XP002141807 page 1360, column 2, paragraph 4 page 1361, column 2, paragraph 3 -page 1363, column 1, paragraph 1 page 1368, column 1, line 21-27	1-8
P,X	URSINI F. ET AL.: "Dual function of the selenoprotein PHGPx during sperm maturation" SCIENCE, vol. 285, 27 August 1999 (1999-08-27), pages 1393-1396, XP002141939 page 1394, column 3, paragraph 3 -page 1395, column 1, paragraph 1	1-8

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/01877

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613225 A	09-05-1996	US 5895749 A	20-04-1999
		AU 4018295 A	23-05-1996
		CA 2203828 A	09-05-1996
		EP 0789538 A	20-08-1997
		JP 11514204 T	07-12-1999
		US 5935800 A	10-08-1999

myoglobin which possesses a very slow rate of autoreduction; (3) its formation is relatively easy, requiring only metmyoglobin and H_2O_2 solutions; (4) its concentration can be assessed reliably by absorption spectroscopy; and (5) the latter property permits indirect quantification of antioxidant molecules consumed and facilitates calculation of rate constants.

[20] Enzymatic and Immunological Measurements of Soluble and Membrane-Bound Phospholipid-Hydroperoxide Glutathione Peroxidase

By ANTONELLA ROVERI, MATILDE MAIORINO, and FULVIO URSINI

Introduction

Phospholipid-hydroperoxide glutathione peroxidase (PHGPx, EC 1.11.1.12) is a selenoenzyme in which selenium is present in the active site as selenocysteine.[1] The TGA codon, which specifies for the [Se]Cys incorporation in polypeptide chains,[2,3] has been identified in the cDNA sequence.[4] PHGPx has been purified to homogeneity from several sources, namely, pig heart, liver, and brain,[1,5] rat testis and liver, and human placenta (A. Roveri, M. Maiorino, and F. Ursini, unpublished).

PHGPx reduces phospholipid hydroperoxides as well as small soluble hydroperoxides.[6] It has been observed that PHGPx is also competent for the reduction of cholesterol and cholesterol ester hydroperoxides in liposomes, membranes, and oxidized low-density lipoproteins.[7,8] This capability to react with different hydroperoxides accounts for the evidence that all titrable hydroperoxide groups generated in microsomal membranes during lipid peroxidation are reduced by this enzyme, while being resistant

[1] M. Maiorino, C. Gregolin, and F. Ursini, this series, Vol. 186, p. 448 (1990).
[2] A. Böck, K. Forchhammer, J. Heider, and C. Baron, *Trends Biol. Sci.* **16**, 463 (1991).
[3] R. A. Sunde, *Annu. Rev. Nutr.* **10**, 451 (1990).
[4] R. Schuckelt, R. Brigelius-Flohe', M. Maiorino, A. Roveri, J. Reumkens, W. Strassburger, F. Ursini, B. Wolf, and L. Flohe', *Free Radical Res. Commun.* **14**, 343 (1991).
[5] F. Ursini, M. Maiorino, M. Valente, L. Ferri, and C. Gregolin, *Biochim. Biophys. Acta* **710**, 197 (1982).
[6] F. Ursini, M. Maiorino, and C. Gregolin, *Biochim. Biophys. Acta* **839**, 62 (1985).
[7] J. P. Thomas, M. Maiorino, F. Ursini, and A. W. Girotti, *J. Biol. Chem.* **265**, 454 (1990).
[8] J. P. Thomas, P. G. Geiger, M. Maiorino, F. Ursini, and A. W. Girotti, *Biochim. Biophys. Acta* **1045**, 252 (1990).

to classic glutathione peroxidase (GPx).[9] The observation that microsomal lipid peroxidation is inhibited by PHGPx and glutathione only if the membranes contain a physiological amount of vitamin E suggests a tandem mechanism for the two antioxidant activities.

During microsomal lipid peroxidation peroxy radicals are produced, which, by reacting with other lipids, generate carbon-centered radicals, thereby propagating peroxidation and producing lipid hydroperoxides. Metal ion-catalyzed reductive decomposition of the hydroperoxides generates alkoxy radicals able to start new peroxidation chains. By reacting with lipid peroxy radicals, vitamin E prevents propagation and also generates lipid hydroperoxides, which if reduced by PHGPx do not give rise to alkoxy radicals. PHGPx, therefore, prevents the formation of new peroxidation chains and spares vitamin E.[10]

It has long been known that selenium is specifically required for normal spermatogenesis[11] and that its concentration in testis is carefully controlled by a homeostatic mechanism, leading to a priority of supplementation over other organs.[12] A high PHGPx activity has been observed in adult rat testes, mostly linked to the nuclear and mitochondrial fractions.[13] Moreover, the enzyme is expressed only after puberty, disappears after hypophysectomy, and is partially restored by gonadotropin treatment, suggesting its involvement in cellular proliferation and/or differentiation.

Factors addressing PHGPx in the membrane or soluble compartment at present are not known. Membrane-bound PHGPx from testes shows the same substrate specificity, electrophoretic mobility, immunological reactivity, and peptide mapping with cyanogen bromide and SV-8 protease with respect to the cytosolic form (A. Roveri, M. Maiorino, and F. Ursini, unpublished). In organs other than adult testes, membrane-bound PHGPx, although identifiable by immunological techniques, is hardly detectable by enzymatic assays. For this reason, which is possibly related to modulation of enzyme activity *in vivo*, it is advisable to evaluate PHGPx in terms of both specific activity and protein.

Materials

Glutathione reductase (EC 1.6.4.2), type IV from bakers' yeast; β-nicotinamide adenine dinucleotide phosphate, reduced form

[9] M. Maiorino, A. Roveri, F. Ursini, and C. Gregolin, *J. Free Radicals Biol. Med.* **1**, 203 (1985).
[10] M. Maiorino, M. Coassin, A. Roveri, and F. Ursini, *Lipids* **24**, 721 (1989).
[11] A. S. H. Wu, J. E. Oldfield, L. R. Shull, and P. R. Cheeke, *Biol. Reprod.* **20**, 793 (1979).
[12] D. Behne, T. Höfer, R. von Berswordt-Wallrabe, and W. Elger, *J. Nutr.* **112**, 1682 (1982).
[13] A. Roveri, A. Casasco, M. Maiorino, P. Dalan, A. Calligaro, and F. Ursini, *J. Biol. Chem.* **267**, 6142 (1992).

(β-NADPH); glutathione, reduced form (GSH); L-α-phosphatidylcholine, type III-S from soybean; and lipoxidase (EC 1.13.11.12), type IV from soybean, are purchased from Sigma Chemical Company (St Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) and pepstatin A are from Serva Feinbiochemica GmbH (Heidelberg, Germany).

Leupeptin hemisulfate and 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) are from Fluka Chemie AG (Buchs, Switzerland). Ethylenediaminetetraacetic acid, disodium salt (EDTA), is from Merck (Darmstadt, Germany).

Biotinylated anti-rabbit immunoglobulin (Ig) and streptoavidin–alkaline phosphatase complex are from Amersham International plc (Amersham, UK). Triton X-100, 4-nitrophenyl phosphate disodium salt, 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and 4-nitro blue tetrazolium chloride (NBT) are from Boehringer Mannheim GmbH (Mannheim, Germany). Dynatech microtiter plates (Cat. No. M 129 A) are purchased from PBI International (Milan, Italy).

Sample Preparation

Fresh tissues are minced in ice-cold 0.1 *M* Tris-HCl, 0.25 *M* sucrose, pH 7.4, containing 5 m*M* 2-mercaptoethanol, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, and 100 μg/ml PMFS and washed twice. Tissues are then homogenized in a Potter homogenizer in 3 volumes of the same buffer. Fibrous parts, unbroken cells, and cell debris are eliminated by centrifugating at 500 *g* for 5 min. Supernatants can be used either as whole homogenates or separated into subcellular fractions by differential centrifugation. Nuclei, mitochondria, microsomes, and cytosol are obtained by centrifugating at 2000 *g* for 10 min, at 20,000 *g* for 30 min, and at 105,000 *g* for 1 hr, respectively. When just small samples are available, total membrane and soluble fractions are separated by centrifugating the whole homogenate at 105,000 *g* for 1 hr. Pellets are washed in homogenization buffer, respun, and resuspended to a final concentration of 10–20 mg protein/ml.

Activity Measurement

Enzymatic activity is measured in a coupled test, by using nonlimiting amounts of glutathione reductase as ancillary enzyme and saturating amounts of NADPH[14] and phosphatidylcholine hydroperoxides as sub-

[14] R. Heiner Schirmer, R. Luise Krauth-Siegel, and G. E. Schultz, *in* "Coenzymes and Cofactors" (D. Dolphin, R. Poulson, and O. Avramovic, eds.). Vol. 3, Part A, p. 553. Wiley (Interscience), New York, 1989.

strate.[6] Activity is measured spectrophotometrically at 340 nm from the specific rate of NADPH oxidation.

The substrate is prepared by enzymatic hydroperoxidation of phosphatidylcholine by soybean lipoxidase type IV[1,15]: 22 ml of 0.2 *M* Tris-HCl, pH 8.8, containing 3 m*M* sodium deoxycholate and 0.3 m*M* phosphatidylcholine is incubated at room temperature, under continuous stirring, for 30 min with 0.7 mg of soybean lipoxidase type IV. The mixture is loaded on a Sep-Pak C_{18} cartridge (Waters–Millipore, Milford MA) washed with methanol and equilibrated with water. After washing with 10 volumes of water, phosphatidylcholine hydroperoxides are eluted in 2 ml of methanol.

Traces of deoxycholate are always present in the methanolic solution of phosphatidylcholine hydroperoxides. This must be pointed out because, in the presence of Triton X-100, deoxycholate can stimulate PHGPx activity, while in the absence of the detergent it is inhibitory.[16] When different samples have to be compared, it is, therefore, convenient to use the same batch of substrate. Methanolic solutions of phosphatidylcholine hydroperoxides (PC-OOH) are stable for months at −20°.

The spectrophotometric test mixture contains, in 2.5 ml, the following: 0.1 *M* Tris-HCl, pH 7.4, 3 m*M* GSH, 10 m*M* NaN_3, 5 m*M* EDTA, 1.5 IU glutathione reductase, 0.1% (v/v) Triton X-100, and 0.2 m*M* NADPH. Samples (0.5–1.5 mg/ml) are added to the test mixture and incubated for some minutes at 37° to allow the enzyme and glutathione to be converted to the reduced forms. The nonspecific NADPH oxidation rate is recorded for 0.5–2 min, and then the enzymatic reaction is started by the addition of PC-OOH (10–30 μM). Activity is calculated by subtracting the nonspecific oxidation rate from the observed NADPH oxidation rate after the substrate addition.

To rule out the presence in crude samples of substances interfering with PHGPx activity, it is advisable to repeat the test with different amounts of enzyme-containing sample and, if purified PHGPx is available, to check that the activity is not affected by the presence of the sample itself. Two criteria can be applied to confirm the specificity of the attribution to PHGPx of the observed activity. (1) To judge the extent of the reaction, the nanomoles of NADPH oxidized during the test should be stoichiometric to the nanomoles of PC-OOH added at the beginning of the reaction. Thus, either an early arrest of the reaction or a drift of the NADPH oxidation rate must be considered suspect. (2) Specificity can be confirmed by iodoacetate inhibition: PHGPx and GPx activities are completely inhibited

[15] J. Eskola and S. Laakso, *Biochim. Biophys. Acta* **751**, 305 (1983).
[16] M. Maiorino, A. Roveri, C. Gregolin, and F. Ursini, *Arch. Biochem. Biophys.* **251**, 600 (1986).

after incubation with 2 m*M* sodium iodoacetate in the presence of 3 m*M* thiols. At the pH used, only selenol moieties involved in the catalytic cycle are dissociated and are reactive with iodoacetate.[17] Samples are incubated in the reaction mixture containing also 2 m*M* sodium iodoacetate at 37° for 10 min. The activity still present on the addition of PC-OOH is subtracted from activity measured as usual.

Enzymatic activity can also be evaluated fluorimetrically by recording the rate of NADPH oxidation, using wavelengths of 350 and 450 nm for excitation and emission, respectively.

Immunoenzymatic Determination

PHGPx as protein is measured by an enzyme-linked immunosorbent assay (ELISA) procedure using rabbit anti-pig heart PHGPx polyclonal antibodies and purified pig heart PHGPx as standard.[18] The test is a competition ELISA in which antigen bound on the surface of the well competes with antigen in solution (known amounts of purified PHGPx for the calibration curve or unknown amounts for samples) for the antibody.

Production of Antibodies

Pig heart PHGPx is purified to homogeneity as previously described in this series.[1] Further purification is achieved by preparative gel electrophoresis according to Laemmli[19] in a $T = 12\%$, $C = 2.67\%$ separating gel. About 200 μg of semipure PHGPx is loaded and run in the gel. After staining with Coomassie Brilliant Blue R-250 (CBB), the band of PHGPx is cut from the whole gel and exhaustively dialyzed against phosphate-buffered saline (PBS), pH 7.4, at 4°. Gel pieces are then homogenized in as small a volume of PBS as possible in a Potter homogenizer. The homogenate is mixed with an equal volume of Freund's complete adjuvant in order to obtain a stable emulsion. This is eventually injected into the back of a 2-month-old New Zealand rabbit at about 50 injection sites, especially along the backbone, near the neck, and near the upper part of the forelimbs and hindlimbs. Booster injections are made intramuscularly in the forelimbs and hindlimbs after 4, 8, and 12 weeks; in this case the protein to be injected is prepared as described above, but Freund's incomplete adjuvant is used.[20]

[17] J. Chaudiere and A. L. Tappel, *Arch. Biochem. Biophys.* **226**, 448 (1983).
[18] E. Harlow and D. Lane, "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.
[19] U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
[20] J. L. Vaitukaitis, this series, Vol. 73, p. 46.

Serum collected 2 weeks after the last booster and tested by Western blot analysis recognizes PHGPx on nitrocellulose up to a dilution of 1 : 4000. Preimmune serum, as well as immune serum adsorbed with purified PHGPx, fails to detect the protein. Immune serum raised against pig heart PHGPx recognizes this enzyme in different rat, pig, mouse, and human tissues. The antiserum is not cross-reactive to classic GPx from any source.

Standard Enzyme

The protein concentration in PHGPx preparations is determined according to Bradford, using bovine serum albumin (BSA) as the standard protein. Different quantities of each preparation (from 2.5 to 15 μg total protein) are run in a $T = 16.5\%$ and $C = 3\%$ separating gel according to Schägger and von Jagow[21] and stained with CBB. By densitometric scanning of electrophoretograms, the relative percentage of the PHGPx band with respect to all the others is determined to obtain a more accurate estimation of the amount of PHGPx. Homogeneous preparations of PHGPx are used in ELISA test, both for the coating of the ELISA plate and for the calibration curve.

Coating of Plates

Polystyrene plates are from Dynatech. In our experience, the most efficient buffer for coating wells with PHGPx is 0.1 *M* sodium carbonate buffer, pH 9.6. Coating is performed overnight at 4°.

Assay Preparation

To determine the best conditions for maximal sensitivity in the ELISA, different amounts (2.5 to 50 ng in 100 μl of carbonate buffer) of PHGPx are coated on each well. Dilutions of immune serum, ranging from 1 : 1000 to 1 : 8000, are tested for each amount of PHGPx. The antigen–antibody reaction is detected as absorbance at 405 nm following the procedure described later.

The amount of PHGPx giving a signal close to saturation in the presence of the lowest serum dilution is chosen for coating. The serum dilution to be used in the ELISA test is then chosen within the linear part of the plot of serum dilutions versus absorbance in wells coated with amounts of PHGPx identified as above. In our experience the optimal conditions are 10.5 ng of PHGPx for coating and a dilution of 1 : 2000 of antiserum.

[21] H. Schagger and G. von Jagow, *Anal. Biochem.* **166**, 368 (1987).

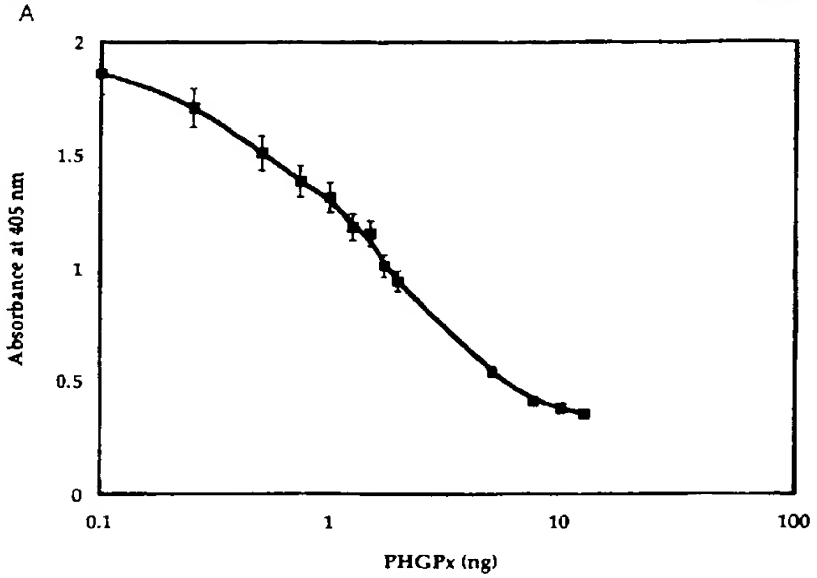


FIG. 1. (A) Semilogarithmic plot of PHGPx versus absorbance in the ELISA test. (B) Plot of the logarithm of $(A_m - A)/A$ versus the logarithm of PHGPx in solution, where A_m is the absorbance in the absence of PHGPx and A is the absorbance in the presence of different amounts of PHGPx in solution (see text for details).

Assay Procedure

Plate coating and adsorption of antiserum with standard PHGPx or samples are carried out overnight at 4°. Standard PHGPx is diluted in 0.1 *M* sodium carbonate buffer, pH 9.6, to get a final concentration of 10.5 ng in 100 μl and loaded. Blank wells are coated with 100 μl of carbonate buffer.

For the standard curve, different amounts of PHGPx (from 0.1 to 12.5 ng) are diluted to 50 μl in homogenation buffer and then diluted 1 : 1 with serum diluted 1 : 1000 in PBS containing 1% BSA, so that the final serum dilution is 1 : 2000. Each point is repeated four times.

Eight dilutions (from 0.1 to 7.5 μl) of the sample are tested with the corresponding blank (i.e., using wells without any PHGPx) in quadruplicate. Each sample is first diluted to 50 μl in homogenation buffer and then 1 : 1 with diluted serum as for the calibration curve. When dealing with total homogenate or membranes, 24 m*M* CHAPS is added to homogenation buffer both in the calibration curve and in the samples. This is critical for

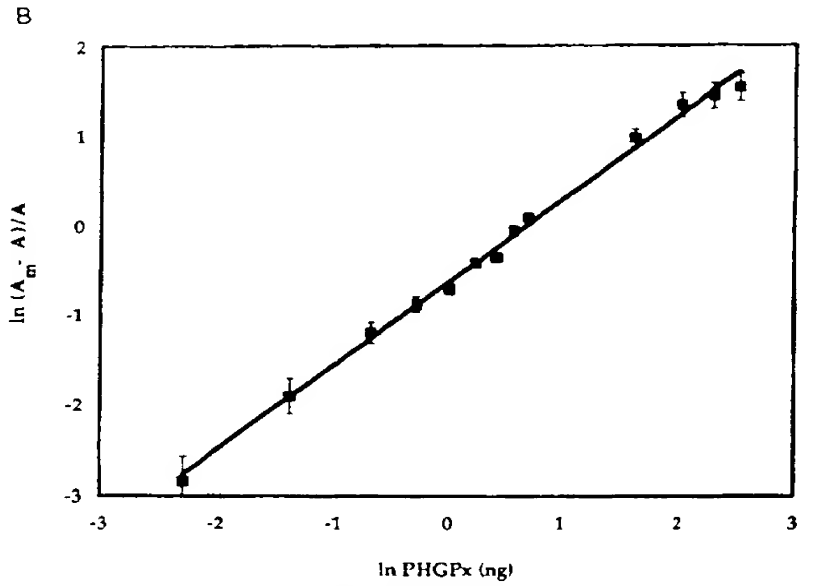


FIG. 1. (*continued*)

detection of PHGPx in brain membranes. In this case the sample is first incubated for 1 hr at room temperature in the homogenization buffer containing the detergent. A protein concentration in the sample ranging from 10 to 20 mg/ml is advisable for an optimal lipid to detergent ratio of sample dilutions used for the ELISA. After diluting with serum, samples are kept overnight at 4°.

The day after wells are saturated with 250 μl of 3% (w/v) BSA in 0.9% (w/v) NaCl for 2 hr at 37°, then they are washed once with PBS containing 0.1% (v/v) Tween 20 and then incubated with 100 μl of adsorbed serum for 1 hr at 37°. After incubation with the adsorbed serum, wells are washed three times with PBS, 0.1% (v/v) Tween 20 and then incubated with 100 μl of biotinylated anti-rabbit Ig diluted in PBS, 1% (w/v) BSA, as suggested by the manufacturer, for 1 hr at 37°. Wells are then washed again and incubated with 100 μl of streptavidin–alkaline phosphatase complex diluted in PBS, 1% BSA, 0.1% (v/v) Tween 20, as suggested by the manufacturer, for 30 min at 37°. After washing again, the phosphatase reaction is started by the addition of 100 μl of substrate buffer (1 mg/ml 4-nitrophenylphosphate in 0.1 *M* diethanolamine hydrochloride, 1 m*M*

TABLE I
PHOSPHOLIPID-HYDROPEROXIDE GLUTATHIONE PEROXIDASE ACTIVITY IN SOLUBLE AND MEMBRANE FRACTIONS OF RAT TISSUES

Tissue	Soluble fraction (nmol/min/mg protein)	Membrane fraction (nmol/min/mg protein)
Adrenals	11.49 ± 2.04	Undetectable
Brain	1.97 ± 0.78	Undetectable
Heart	3.59 ± 1.63	4.41 ± 1.65
Kidney	9.87 ± 0.11	Undetectable
Liver	6.09 ± 0.36	4.00 ± 0.34
Lung	2.10 ± 0.97	4.72 ± 2.67
Muscle (sartorius)	Undetectable	Undetectable
Ovary	8.96 ± 3.05	Undetectable
Spleen	6.69 ± 3.02	Undetectable
Testis	32.21 ± 3.60	158.70 ± 18.56
Thymus	Undetectable	Undetectable
Thyroid	7.27 ± 1.36	Undetectable
Uterus	Undetectable	Undetectable

$MgCl_2$, pH 9.8). After 30 min in the dark, at room temperature, the reaction is stopped with 100 μl of 3 *M* NaOH. The absorbance at 405 nm is recorded using a microplate reader (Bio-Rad, Richmond, CA, model 450 microplate reader), after calibrating the instrument against a blank.

Data Processing

Mean absorbance and standard deviation (S.D.) are measured for each sample and the corresponding blank. The difference between the absorbance of the sample and the blank is the actual absorbance of the sample under investigation.

The plot of the logarithm of nanograms of PHGPx versus absorbance appears as a sigmoid shaped curve. To obtain a linear plot, data are processed as follows. Absorbances measured after incubation with different amounts of PHGPx (*A*) are subtracted from the absorbance obtained in the absence of any added PHGPx (A_m). Each value is then divided by *A* and eventually the logarithm of this ratio is plotted versus the logarithm of the nanograms of PHGPx (Fig. 1).

Data obtained using unknown samples are processed as above, and the amount of PHGPx is calculated by using the standard curve. For each sample eight dilutions are used. Data obtained with this procedure are reproducible and reliable, and they have been qualitatively confirmed by Western blotting analysis.

TABLE II
PHOSPHOLIPID-HYDROPEROXIDE GLUTATHIONE PEROXIDASE CONTENT IN SOLUBLE AND MEMBRANE FRACTIONS OF RAT TISSUES

Tissue	Soluble fraction (ng/mg protein)	Membrane fraction (ng/mg protein)
Adrenals	4.78 ± 1.19	8.83 ± 4.01
Brain	2.98 ± 1.56	6.91 ± 3.10
Heart	1.26 ± 0.69	0.96 ± 0.26
Kidney	1.60 ± 1.04	4.28 ± 2.31
Liver	3.46 ± 1.67	15.88 ± 5.67
Lung	1.47 ± 0.30	2.65 ± 0.83
Muscle (sartorius)	1.01 ± 0.50	30.50 ± 8.30
Ovary	1.83 ± 0.28	5.40 ± 1.83
Spleen	1.26 ± 0.38	3.76 ± 0.42
Testis	2.99 ± 0.38	53.60 ± 9.10
Thymus	1.02 ± 0.49	21.04 ± 9.94
Thyroid	6.77 ± 3.24	5.37 ± 2.64
Uterus	2.68 ± 1.11	6.87 ± 0.99

Western Blotting

Samples (whole homogenate or subcellular fractions) are dried in a Speed-Vac and then solubilized in sample buffer[19] to a final protein concentration of 4 mg/ml. Electrophoresis is carried out according to Laemmli[19] in a $T = 12\%$, $C = 2.67\%$ separating gel. Usually 100 μg of each sample is loaded on each well. Purified PHGPx (100 ng) is used as the standard and positive control for Western blotting.

After electrophoresis, the gel is equilibrated in transfer buffer (25 m*M* ethanolamine, 104 m*M* glycine, and 20% (v/v) methanol, pH 9.5[22] and then blotted onto a nitrocellulose membrane (Bio-Rad nitrocellulose membrane, 0.2 μm). Protein blotting is performed at the anode overnight, at 20°, at 200 mA constant current, in a tank-type transfer system (Hoefer Transphor Transfer Electrophoresis Unit). Blotting is checked by staining with 0.3% (w/v) Ponceau Red S, in 2% (w/v) trichloroacetic acid.

Residual sites for protein binding on nitrocellulose are saturated with 3% BSA, 0.1% Tween 20 in 0.9% NaCl for at least 2 hr. The membrane is then incubated with rabbit immune serum diluted (1 : 1000) in PBS, 1% BSA for 1 hr. After washing three times with PBS, 1% BSA, and 0.1% Tween 20, the membrane is allowed to react with the biotinylated anti-rabbit Ig, and, after washing again, with the streptavidin–alkaline phosphatase complex. Eventually, after the last washing, the alkaline phosphatase

[22] B. Szewczyk and L. M. Kozloff, *Anal. Biochem.* **150**, 403 (1985).

reaction is started in 100 ml of 0.1 *M* Tris-HCl, pH 9.5, 0.1 *M* NaCl, 5 m*M* $MgCl_2$, 15 mg X-phosphate (in 1 ml of dimethylformamide), and 30 mg NBT (in 1 ml of 70% (v/v) dimethylformamide). The reaction is stopped in dilute HCl. The negative control is carried out by substituting immune serum adsorbed with purified PHGPx for immune serum.

Conclusions

By using the described procedure, PHGPx activity can be measured in several tissues. The critical points are the substrate preparation, the stoichiometry between the amount of peroxidic substrate used and NADPH oxidized, and the use of a blank in the presence of iodoacetate for absorbance measurements. The distribution of the activity in membranes and in the soluble fraction of rat tissues, obtained by the described procedure, is reported in Table I.

The ELISA test is accurate and very sensitive. Furthermore, the possibility of measuring the enzyme as protein instead of as activity could allow the identification of a modulation of the catalytic capacity by possible posttranslational modifications of the enzyme. This seems to be more than an appealing possibility since the amount of the protein PHGPx in soluble and membrane fractions of rat tissues (Table II) does not fit the activity distribution. In almost all tissues a substantial amount of PHGPx is present in membranes in a scarcely active form. Although the mechanism(s) for activation–inactivation is unknown, the evidence of very active enzyme in membranes of spermatogenic cells undergoing differentiation highlights a possible relationship between peroxide level in these membranes and differentiation or proliferation, extending the area of PHGPx involvement beyond antioxidant protection.

[21] Targeting Superoxide Dismutase by Gene and Protein Engineering

By MASAYASU INOUE

Introduction

Protection of tissues from oxygen toxicity is one of the major prerequisites to aerobic life.[1] Reactive oxygen species rapidly react

[1] H. Sies (ed.), "Oxidative Stress." Academic Press, New York, 1985.

This conclusion is especially true in view of the fact that the physiological P_{O_2} in liver ranges from 1 to 60 mmHg.[12] Further instances of the importance of the actual P_{O_2} in the formation of reactive O_2 species are Adriamycin-induced lipid peroxidation, where recently an O_2 dependence of lipid peroxidation similar to that described for haloalkane-dependent lipid peroxidation has been reported,[13] and the formation of superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) by redox cycling of menadione, where at physiological P_{O_2} the electron transfer from the reduced forms of this quinoid compound to the mitochondrial respiratory chain appears to dominate over the reduction of molecular oxygen.[14]

Acknowledgments

Stimulating discussions with Prof. H. Sies are gratefully acknowledged. Research was supported by Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen.

[12] M. Kessler, J. Höper, D. K. Harrison, K. Skolasinska, W. P. Klövekorn, F. Sebening, H. J. Volkholz, I. Beier, C. Kernbach, V. Rettig, and H. Richter, *in* "Oxygen Transport to Tissue" (D. W. Lübbers, H. Acker, E. Leniger-Follert, and T. K. Goldstick, eds.), Vol. 5, p. 69. Plenum, New York, 1984.
[13] G. F. Vile and C. C. Winterbourn, *Biochem. Pharmacol.* **37**, 2893 (1988).
[14] H. de Groot, T. Noll, and H. Sies, *Arch. Biochem. Biophys.* **243**, 556 (1985).

[47] Phospholipid Hydroperoxide Glutathione Peroxidase

By MATILDE MAIORINO, CARLO GREGOLIN, and FULVIO URSINI

Introduction

Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is the second selenoenzyme discovered in mammals.[1] The enzyme was first identified and purified by following its antiperoxidant activity, and for this reason it was originally named peroxidation-inhibiting protein (PIP).[2] This enzyme was purified to homogeneity from pig liver,[2] heart,[3] and brain[4] and rat liver,[2] and it has been identified in all tissues in which it was

[1] F. Ursini, M. Maiorino, and C. Gregolin, *Biochim. Biophys. Acta* **839**, 62 (1985).
[2] F. Ursini, M. Maiorino, M. Valente, and C. Gregolin, *Biochim. Biophys. Acta* **710**, 197 (1982).
[3] M. Maiorino, P. Ursini, M. Leonelli, N. Finato, and C. Gregolin, *Biochem. Int.* **5**, 575 (1982).

searched for namely, rat kidney, heart, lung, muscle, and brain,[5,6] dog liver brain and kidney, bull spermatozoa, and fish liver (unpublished). A high activity has been recently observed in rat testis (unpublished). The chromatographic behavior of the protein and purification procedures were identical for all tissues from which it was purified.

Because PHGPX is a soluble enzyme, which has been purified from cell sap it can be classified as a cytosolic enzyme. However, a substantial activity, which can be partially recovered by high ionic strength extraction,[5] is present in membranes of subcellular organelles. Based on present knowledge, therefore, this enzyme must be regarded as a cytosolic enzyme that is active on membranes to which it is bound to some extent. Immunohistochemical localization of the enzyme will clarify this issue.

(PHGPX) is a monomeric enzyme (MW ~20,000 on SDS–PAGE) that contains one selenium atom at the active site. The selenoprotein nature of PHGPX was assessed by proton-induced X-ray fluorescence of the purified protein. The presence of a selenocysteinwas first suggested by inhibition kinetics in the presence of iodoacetate and thiols, and it was subsequently demonstrated by amino acid analysis following carboxymethylation and acidic digestion (L. Flohé, personal communication).

PHGPX reduces the hydroperoxy derivatives of phospholipids to alcohol derivatives, and this activity is absent both in Se-dependent tetrameric glutathione peroxidase and in glutathione transferase B (the so-called Se-independent glutathione peroxidase). PHGPX is therefore unique in that it reduces the hydroperoxides of the major components of membranes. The reduction of phospholipid hydroperoxides was demonstrated by HPLC and mass spectroscopic analysis of intact phospholipid substrate and product[7] and by HPLC analysis of the fatty acid derivatives released by enzymatic hydrolysis.[8]

The specificity for the peroxide substrate is broad, and the enzyme is active on all phospholipid hydroperoxides, as well as fatty acid hydroperoxides, cumene hydroperoxide, *tert*-butyl hydroperoxide, and hydrogen peroxide. The wide specificity for hydroperoxides is also suggested by

[5] F. Ursini, M. Maiorino, L. Bonaldo, and C. Gregolin, *in* "Oxygen Radicals in Chemistry and Biology" (W. Bors, M. Saran, and D. Tait, eds.), p. 713. de Gruyter, Berlin and New York, 1984.

[6] F. Ursini, M. Maiorino, and C. Gregolin, *in* "Oxy Radicals and Their Scavenger System" (R. A. Greenwald and G. Cohen, eds.), Vol. 2, p. 224. Elsevier, New York, 1983.

[7] L. Zhang, M. Maiorino, A. Roveri, and F. Ursini, *Biochim. Biophys. Acta* **1006**, 140 (1989).

[8] S. Daolio, P. Traldi, F. Ursini, M. Maiorino, and C. Gregolin, *Biomed. Mass Spectrom.* **10**, 499 (1983).

[9] F. Ursini, L. Bonaldo, M. Maiorino, and Gregolin, *J. Chromatogr.* **270**, 301 (1983).

evidence that all titratable hydroperoxide groups generated in microsomal membranes during lipid peroxidation are reduced by this enzyme.[9] Furthermore, it has recently been observed directly that PHGPX also reduces cholesterol hydroperoxides.[10] The physiological reducing substrate is glutathione, although inhibition experiments in the presence of iodoacetate suggest that the oxidized active site can also be reduced by mercaptoethanol.[1]

The kinetic mechanism of PHGPX appears identical to that of glutathione peroxidase. Kinetic analysis, indeed, was compatible with a Ping-Pong mechanism without the formation of ternary complexes.[1] In this mechanism, as in the case of glutathione peroxidase, the kinetic parameters for one substrate (V_{max} and K_m) are not defined, being a function of the other substrate. In the suggested reaction scheme, the selenol moiety of PHGPX is first oxidized by hydroperoxides and then reduced back in two steps by two glutathione molecules. This mechanism exactly fits the Dalziel equation and the integrated rate equation describing the Tert-Uni Ping-Pong mechanism that have been applied to glutathione peroxidase[11,12] From the plots obtained by computer processing of the progression curves of the activity, it is possible to calculate the pseudo-first-order rate constant for the first reaction of the catalytic cycle, i.e., the reduction of the hydroperoxide and the oxidation of the selenol. This parameter was measured on different substrates, and the results were compared with those obtained using tetrameric glutathione peroxidase.[13] The results showed that glutathione peroxidase is much more active than PHGPX on hydrogen peroxide, that both enzymes react almost equally well on linoleic acid hydroperoxide, and that only PHGPX is active on phospholipid hydroperoxides (Table I). These kinetic data indicate that an identical kinetic mechanism has been adopted by nature to control the hydroperoxide concentration in different environments: one enzyme is active on small hydrophilic substrates and the other on lipophilic substrates in membranes.

The identical kinetic mechanisms of the peroxidase reactions, the selenium content, and the similarity of molecular weight between PHGPX

[9] M. Maiorino, A. Roveri, F. Ursini, and C. Gregolin, *J. Free Radicals Biol. Med.* **1**, 203 (1985).

[10] J. P. Thomas, M. Maiorino, F. Ursini, and A. W. Girotti, *J. Biol. Chem.* **454**, in press (1990).

[11] L. Flohé, G. Loeschen, W. A. Gunzler, and E. Eichele, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 987 (1972).

[12] J. Chaudière and A. L. Tappel, *Arch. Biochem. Biophys.* **226**, 448 (1983).

[13] F. Ursini and A. Bindoli, *Chem. Phys. Lipids* **44**, 255 (1987).

TABLE I
APPARENT SECOND-ORDER RATE CONSTANTS FOR REACTION BETWEEN PHGPX OR GPX AND HYDROPEROXIDE SUBSTRATES[a]

Substrate	K (mM^{-1} min^{-1})	
	PHGPX	GPX
Hydrogen peroxide	1.9×10^3	2.9×10^6
Cumene hydroperoxide	1.3×10^5	1.0×10^6
tert-Butyl hydroperoxide	7.1×10^4	7.1×10^5
Linoleic acid hydroperoxide	1.8×10^6	2.3×10^6
Phosphatidylcholine hydroperoxide	7.0×10^5	—

[a] From Refs. 1 and 12.

and the monomer of glutathione peroxidase might seem to suggest a structural relationship between these enzymes. However, the identification of PHGPX with the monomer of GPX seems to be excluded for the following reasons: (1) the amino acid composition is different (even though the amino acid composition of pig heart glutathione peroxidase is not available, and the comparison was made between bovine red blood cell glutathione peroxidase and pig heart PHGPX); (2) the monomer of glutathione peroxidase is catalytically inactive, independent of the substrate; and (3) there is a completely different susceptibility to inhibitors.[14]

PHGPX was identified and subsequently purified as a cytosolic protein that inhibits microsomal lipid peroxidation in the presence of glutathione. The first apparent role of this enzyme in cell biology, therefore, seems to be related to the protection of biomembranes against oxidative damage.[2] During microsomal lipid peroxidation, lipid hydroperoxides are produced. The decomposition of lipid hydroperoxides generates the extremely reactive alkoxyl radical which, in turn, generates new hydroperoxides. The enzymatic reduction of the hydroperoxides prevents this free-radical multiplication. However, the catalytic activity of PHGPX cannot account per se for the almost complete protection observed in microsomes undergoing peroxidation. The inhibition of lipid peroxidation, induced by iron and reducing equivalents (ascorbate or NADPH), by PHGPX and glutathione requires a physiological amount of vitamin E in membranes, suggesting a tendem mechanism for the antioxidant activities

[14] M. Maiorino, A. Roveri, C. Gregolin, and F. Ursini, *Arch. Biochem. Biophys.* **251**, 600 (1986).

of PHGPX and vitamin E.[15] Reacting vitamin E with lipid hydroperoxyl radicals prevents propagation of peroxidation but generates lipid hydroperoxides. If not promptly reduced by PHGPX, the lipid hydroperoxides undergo decomposition, leading to alkoxy radicals[16] that are extremely reactive against vitamin E and lipids as well.[17] In the absence of PHGPX, vitamin E is just cooxidized with lipids, whereas PHGPX without vitamin E can only slow down the free-radical multiplication rate, and its capacity is rapidly saturated.

Although protection against lipid peroxidation is the most prominent function of the enzyme, it is plausible that PHGPX would also affect the physiological control of hydroperoxides enzymatically generated, and that these hydroperoxides would affect cellular functions or activation. In this case a new, exciting chapter on the physiology of PHGPX could begin.

Substrate Preparation and Activity Measurement

Taking advantage of its peroxidation-inhibiting activity, PHGPX was discovered and purified in a simple lipid peroxidation test. The peroxidase activity, which was first identified on a partially purified preparation of the enzyme, was in fact measurable in crude fractions with some difficulties. Thereafter, the introduction, as peroxide substrate, of mixed micelles of phospholipid hydroperoxides and Triton X-100 greatly simplified activity measurements. PHGPX activity, although measurable on liposomes or membranes containing hydroperoxides, is higher and more linear when the substrate is in micellar form. The test we describe can be used throughout all purification steps as well as in tissue homogenates.

Phospholipid hydroperoxides can be prepared either by spontaneous autoxidation or by enzymatic hydroperoxidation of soybean phosphatidylcholine liposomes or dispersions. The enzymatic procedure, which is more practical and reproducible, is described. Polyunsaturated fatty acids of phosphatidylcholine are oxygenated by soybean lipoxidase[18] in the presence of bile salts. At the end of the reaction lipids must be extracted and separated from bile salts, which are strong inhibitors of PHGPX activity.[14]

[15] M. Maiorino, M. Coassin, A. Roveri, and F. Ursini, *Lipids* **24**, 721 (1989).
[16] S. D. Aust and B. A. Svingen, *in* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 5, p. 1. Academic Press, New York, 1982.
[17] M. Erben-Russ, C. Michel, W. Bors, and M. Saran, *J. Phys. Chem.* **91**, 2362 (1987).
[18] J. Eskola and S. Laasho, *Biochim. Biophys. Acta* **751**, 305 (1983).

Enzymatic Hydroperoxidation of Soybean Phosphatidylcholine and Extraction of Products

The reaction is carried out in an oxygraph vial, and the oxygen consumption is recorded. The reaction mixture contains, in 2.5 ml, 0.2 *M* sodium borate (pH 9), 3 m*M* sodium deoxycholate, and 0.3 m*M* soybean phosphatidylcholine. The phospholipids are obtained from Sigma (St. Louis, MO; Type III/S) in chloroform solution. The required amount is dried under an argon stream and first dispersed with 10 m*M* deoxycholate. This dispersion is then diluted with the buffer. The reaction starts by the addition of 0.1 mg of soybean lipoxyganase (EC 1.13.11.12) from Sigma (Type IV). After approximately 10 min, when 50% of the oxygen has been consumed, the mixture is applied to a Sep-Pak C_{18} cartridge (Water Associates, Milford, MA) equilibrated with water. Deoxycholate is washed off with 10 bed volumes of water, and phospholipids are eluted with 2 ml of methanol. This procedure can be scaled up or repeated several times. Methanolic extracts are pooled, and this solution, which contains phospholipid hydroperoxides and native phospholipids, is concentrated to a small volume. It is stable for approximately 1 month at −20°.

Using this procedure the recovery of hydroperoxides accounts for approximately 60% of the oxygen consumed. The amount of hydroperoxide groups can be evaluated from the test described below if partially purified PHGPX is available, or by titration. This preparation of phospholipid hydroperoxides is suitable for the test of PHGPX activity. If necessary, peroxidized phospholipid can be purified by HPLC. A detailed description of the HPLC procedures is not within the scope of this chapter; however, we suggest the procedures described by Crawford *et al.*[19] or Ursini *et al.*[8]

Test of Activity

PHGPX activity is measured spectrophotometrically; glutathione oxidation is recorded at 340 nm in the presence of an excess of glutathione reductase and a saturating amount of NADPH. The peroxide substrates are phospholipid hydroperoxides prepared as described above and dispersed in mixed micellar form in the presence of Triton X-100. Since Triton X-100 might contain some hydroperoxides, it is important to use high-quality, peroxide-free Triton X-100 (Boehringer-Mannheim, West Germany). Moreover, it is convenient to run a blank (without adding lipid hydroperoxides) to search for these Triton X-100 hydroperoxides.

[19] C. G. Crawford, R. D. Plattner, D. J. Sessa, and J. J. Rackis, *Lipids* **15**, 91 (1979).

Reagents

Buffer and reagent solution: 0.5 *M* Tris-HCl (pH 7.4), 25 m*M* EDTA, 0.5 m*M* NADPH, 5 m*M* NaN_3, 15 m*M* reduced glutathione

Glutathione reductase, 5 mg/ml, specific activity 120 U/mg (Boehringer-Mannheim)

Triton X-100 (peroxide free), 20% (v/v) in water

Phospholipid containing hydroperoxide groups in methanol ~15 m*M* containing 0.24 μmol of hydroperoxide groups per micromole of phospholipid)

Assay. To a spectrophotometer cuvette equipped with a magnetic stirrer and a temperature control set at 30°–37° are added 0.5 ml of buffer and reagent mixture, 15 μl of Triton X-100 solution, 5 μl of glutathione reductase, and the sample containing PHGPX activity. The final volume is 2.5 ml. After 5 min for temperature equilibration, complete reduction of glutathione, and PHGPX activation, a baseline is recorded. The reaction is then started by the addition of phospholipid hydroperoxides in methanol. The amount added is usually between 20 and 50 nmol of peroxide groups. The glutathione concentration can range from 0.5 to 5 m*M*. The suggested concentration of 3 m*M* gives a good compromise between activity and unspecific oxidation.

The rate becomes linear in less than 10 sec, suggesting that the time required for the formation of mixed micelles is not a limiting factor under these conditions (identical results are obtained using preformed mixed micelles). The NADPH oxidation is usually followed until all the peroxide substrate is consumed and the baseline slope reaches the original value, before the addition of hydroperoxides. To test that the system is functioning correctly, it is useful to check it by adding a second aliquot of hydroperoxides.

This assay allows for the evaluation of a blank due to nonspecific NADPH and glutathione oxidation (the rate before the addition of hydroperoxides and after their consumption). Under our experimental conditions, glutathione and NADPH oxidation in the presence of hydroperoxides, i.e., the blank without enzyme, is negligible.

If necessary, the reaction can be started by adding PHGPX, but in this case two artifacts are possible. If the enzyme is stored in a solution that does not contain thiols, a progressive increase of the rate of the enzyme reaction is observed, possibly owing to activation of the enzyme (as in the case of glutathione peroxidase). If the solution of PHGPX contains thiols, activation is not required, but some disulfides, which are always present, might lead to an overestimation of the activity. If it is necessary to start the reaction by adding the enzyme, the enzyme should be preincubated with glutathione, glutathione reductase, and NADPH, to avoid these artifacts.

The spectrophotometric test can be utilized for routine purification of the enzyme and for evaluation of activity in tissues. In these cases, to get reproducible results, it is extremely important to check the phospholipid hydroperoxide substrate for hydrolysis. Fatty acid hydroperoxides are indeed substrates for other peroxidases that, if present, lead to an overestimation of the PHGPX activity. It is therefore useful to rule out the hydrolysis of phospholipid hydroperoxides using glutathione peroxidase. Since fatty acid hydroperoxides, but not phospholipid hydroperoxides, are reduced by glutathione peroxidase, if any activity is detected, the substrate is not suitable for PHGPX activity measurement.

PHGPX Activity in Tissues

To search for PHGPX activity in tissues, if activity is low and precise measurements are not possible using homogenates, we use two preparations, neither of them giving the true "total activity" present in the tissue. Nevertheless, measurements obtained by these approaches are more reproducible and useful for comparisons between different samples.

In the first preparation, the tissue is broken in a Polytron homogenizer for 5 min in 3 volumes of 0.1 *M* Tris-HCl (pH 7.4), 0.3 *M* KCl and centrifuged for 15 min at 15,000 *g* and 45 min at 100,000 *g*. The ionic strength of the supernatant is then decreased either by dialysis against 50 m*M* Tris-HCl (pH 7.4) or by chromatography on Sephadex G-25, since we observed that the activity is partially inhibited by the presence of high ionic strength in the test. This preparation contains the cytosolic PHGPX and the enzyme released from all cellular membranes.

In the second preparation, the tissue is homogenized in 3 volumes of 0.25 *M* sucrose, 20 m*M* Tris-HCl (pH 7.4). Following centrifugation for 15 min at 10,000 *g*, Triton X-100 (final concentration of 0.2% v/v) is added to the supernatant to solubilize microsomes. This fraction contains PHGPX from the cytosol and microsomal fraction.

The activity recovered by the latter procedure is approximately 30% higher than that recovered by the first. However, there are more interfering activities, and the optical density of the sample is higher. By the latter procedure the specific activity or rat liver is 6.2 nmol/min/mg protein at 37°. The amount of protein that can be used in this test is approximately 1 mg.

Purification

Purification of PHGPX from pig heart is described; however, the same procedure can also be applied to other organs, e.g., liver or brain. More-

TABLE II
PURIFICATION OF PHGPX FROM PIG HEART

Step	Specific Activity[a]	Units[b]	Recovery (%)
Homogenization, centrifugation, and dialysis	0.002	190	100
DEAE-Sepharose	0.455	130	69
BSP Affinity Chromatography	5.15	90	47
Sephadex G-50	101.23	36	19
HPLC (TSK CM)	310.15	19	10

[a] Specific activity is measured as μmol/min/mg protein at 37°.
[b] One unit of activity catalyzes the reduction of 1 μmol/min at 37°.

over, the procedure can be easily scaled down if only small samples are available. The purification scheme is reported in Table II.

Approximately 500 g of ventricular muscle is prepared from pig heart (from the local slaughterhouse). The tissue is minced and thoroughly homogenized in a Polytron mixer, set at maximum power, for 5 min in approximately 3 volumes of ice-cold 0.1 *M* Tris-HCl (pH 7.4), 5 m*M* 2-mercaptoethanol. The soluble fraction is prepared by two centrifugations of the homogenate: 30 min at 10,000 *g* and 45 min at 100,000 *g*. Before the second centrifugation filter through cheesecloth to eliminate fluffy material. The supernatant is dialyzed exhaustively against 10 m*M* potassium phosphate buffer (pH 7), 5 m*M* mercaptoethanol. During dialysis some proteins precipitate and are eliminated by centrifugation.

The supernatant is applied to a DEAE-Sepharose 6B column (13 × 5.5 cm) equilibrated with 10 m*M* potassium phosphate buffer (pH 7), 5 m*M* mercaptoethanol. The column is washed with 2 liters of the equilibration buffer and then eluted with 0.5 liters of 0.2 *M* potassium phosphate buffer (pH 7), 5 m*M* mercaptoethanol. The active fractions are collected and pooled, and 10% (v/v) (final concentration) glycerol is added. This fraction is loaded on a Sepharose–bromosulfophthalein–glutathione (BSP) affinity column, prepared by linking the bromosulfophthalein–glutathione adduct[20] to CNBr-activated Sepharose.[21] The column is equilibrated with 25 m*M* Tris-HCl (pH 7.2), 5 m*M* mercaptoethanol, 10% (v/v) glycerol. After the sample is loaded, the column is washed with 300 ml of 25 m*M* Tris-HCl (pH 7.2), 100 m*M* KSCN, 5 m*M* mercaptoethanol, 10% (v/v) glycerol. The elution is carried out with 25 m*M* Tris-HCl (pH 7.6), 300 m*M* KSCN, 5 m*M* mercaptoethanol, 10% (v/v) glycerol. Active fractions

[20] G. Whelan, J. Hoch, and B. Combes, *J. Lab. Clin. Med.* **75**, 542 (1970).
[21] S. C. March, I. Parikh, and P. Cuatrecasas, *Anal. Biochem.* **60**, 149, (1974).

are pooled and concentrated to 5–10 ml using an Amicon ultrafiltration apparatus with an YM10 membrane. This fraction is applied on a Sephadex G-50 column (140 × 5.5 cm) equilibrated with 25 m*M* Tris-HCl (pH 7.4), 300 m*M* KSCN, 5 m*M* mercaptoethanol, 10% (v/v) glycerol. The active portion is eluted 160 ml after the void volume of the column. The active fractions are pooled and concentrated with the same ultrafiltration apparatus as before. This fraction is dialyzed against 10 m*M* potassium phosphate (pH 6.5), 0.1 *M* KCl, 5 m*M* mercaptoethanol. Some proteins precipitate during dialysis and are eliminated by centrifugation.

At this purification stage, PHGPX accounts for 40–70% of the proteins. A preparation at this level of purification is useful for routine purposes such as measuring phospholipid hydroperoxides. If PHGPX is stored at this purification step, 10% (v/v) glycerol should be added to the last dialysis buffer. This preparation is very stable (several months at −20°).

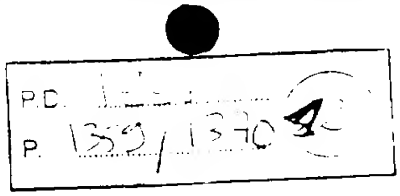
The final purification step is carried out by HPLC using either gel-permeation or ion-exchange columns, e.g., TSK SW 2000 (gel permeation), TSK DEAE (weak anion exchanger), Mono Q (strong anion exchanger), and TSK CM (weak cation exchanger). We describe here the chromatographic conditions for a TSK CM column. Buffer A: 10 m*M* potassium phosphate, 100 m*M* KCl, 5 m*M* mercaptoethanol (pH 6.5); buffer B: 10 m*M* potassium phosphate, 300 m*M* KCl, 5 m*M* mercaptoethanol (pH 6.5). Flow rate: 1 ml/min. The gradient from 0 to 100% buffer B is developed in 25 min after 3 min in isocratic conditions. Detection is at 280 nm; injection volume is less than 0.2 ml. PHGPX is eluted as a single peak when KCl is approximately 200 m*M*. Peaks isolated from several runs are pooled, and 10% (v/v) glycerol is added. The preparation is then concentrated by ultrafiltration to a final protein concentration no higher than 0.3 mg/ml to avoid aggregation.

[48] Iron Redox Reactions and Lipid Peroxidation

By STEVEN D. AUST, DENNIS M. MILLER, and VICTOR M. SAMOKYSZYN

Introduction

Iron-catalyzed lipid peroxidation has been studied in many *in vitro* model systems. While the mechanism of iron-catalyzed lipid peroxidation is not completely understood, it is well established that the redox chemis-



Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation

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ABSTRACT **Selenium deficiency is known to be associated with male infertility, and the selenoprotein PHGPx has been shown to increase in rat testis after puberty and to depend on gonadotropin stimulation in hypophysectomized rats [Roveri et al. (1992) J. Biol. Chem. 267, 6142–6146]. Exposure of decapsulated whole testis, however, failed to reveal any transcriptional activation or inhibition of the PHGPx gene by testosterone, human chorionic gonadotropin, or forskolin. Nevertheless, it was verified that the specific activity of PHGPx in testis, but not of cGPx, correlated with sexual maturation. Leydig cell destruction *in vivo* by ethane dimethane sulfonate (EDS) resulted in a delayed decrease in PHGPx activity and mRNA that could be completely prevented by testosterone substitution. cGPx transiently increased upon EDS treatment, probably as a result of reactive macrophage augmentation. In situ mRNA hybridization studies demonstrated an uncharacteristic low level of cGPx transcription in testis, whereas PHGPx mRNA was abundantly and preferentially expressed in round spermatids. The data show that the age or gonadotropin-dependent expression of PHGPx in testis does not result from direct transcriptional gene activation by testosterone, but is due to differentiation stage-specific expression in late spermatids, which are under the control of Leydig cell-derived testosterone. The striking burst of PHGPx expression at the transition of round to elongated spermatids suggests an involvement of this selenoprotein in sperm maturation.—Maiorino, M., Wissing, J. B., Brigelius-Flohé, R., Calabrese, F., Roveri, A., Steinert, P., Ursini, F., Flohé, L. Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. *FASEB J.* 12, 1359–1370 (1998)**

Key Words: glutathione peroxidases selenium

NUTRITIONAL STUDIES indicate that selenium is essential for male fertility (1–3, 4–6). Its fundamental role in testicular function is corroborated by the observation that, in mild deficiency, selenium is preferentially retained in testis (1). With progressive selenium deficiency, morphological alterations of spermatids and spermatozoa become detectable (2). Extreme deficiency results in the complete disappearance of mature germinal cells (3).

The underlying mechanisms are far from clear; however, at physiological levels, selenium generally exerts its biological functions as selenocysteine residues, which have been cotranslationally incorporated into distinct selenoproteins (7). More than 20 different selenoproteins have been inferred to exist in mammals from pulse labeling experiments with ^{75}Se, but only a minority of them have so far been characterized as distinct gene products or enzymatic entities (8). The latter comprise four members of the glutathione peroxidase family (9–14), at least two deiodinases (15–17), thioredoxin reductase (18), and selenophosphate synthetase (19). Whenever investigated, the selenocysteine moiety of these proteins proved to be essential for catalytic function (20–22) and, correspondingly, the specific enzymatic activities in tissues or whole organisms depend on an adequate supply of selenium in a bioavailable form. Apparently, the biosynthesis of selenoproteins is not only limited by the availability of selenocysteyl-t RNA needed for the incorporation of selenocysteine (7, 23, 24), but can be regulated further at the posttranscriptional level, since specific mRNA species encoding selenoproteins were shown to be stabilized by cellular selenium (reviewed in ref 25).

In the realm of selenoproteins, two glutathione peroxidases are potentially relevant to testicular function. The 'classical' cytosolic glutathione peroxidase

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(cGPx)[1] was reported to be expressed in testis at low levels (26, 27), but has been implicated in the metabolism of H_2O_2 associated with steroid hormone synthesis in Leydig cells (26). Surprisingly high levels of phospholipid hydroperoxide glutathione peroxidase (PHGPx) are found in testis (14, 28), where the enzyme is at least partially directed toward the mitochondria by means of a particular leader sequence. This results from the use of an ATG start codon upstream of the ATG codon more commonly used in other tissues (29). In the rat testis, PHGPx is preferentially expressed after puberty, where it remains essentially absent after hypophysectomy and can be partially restored in hypophysectomized rats by hCG administration (28). Also, immunohistochemical studies revealed an association of PHGPx with the seminiferous epithelium (28). These intriguing observations suggested a transcriptional regulation of PHGPx either by hCG and mediated by cAMP or, indirectly, by testosterone arising from hCG-stimulated Leydig cells, an idea further strengthened by the detection of consensus sequences in the PHGPx gene reminiscent of steroid- and cAMP-responsive elements (11). However, pilot experiments with reporter gene constructs designed to validate the functional relevance of the putative regulatory elements in the known 5′ flanking region of the porcine PHGPx gene are so far inconclusive. Neither testosterone nor forskolin directly activated transcription of the reporter genes nor did estradiol 17-β inhibit transcription in hormone-responsive T47D and MCF7 cell lines (unpublished data).

In the present investigation, we rule out any short-term transcriptional regulation of PHGPx gene expression by testosterone, estrogen, or cAMP. Instead, we demonstrate that the marked increase of PHGPx activity with sexual maturation parallels the hormone-dependent increase of the spermatid layer that preferentially expresses PHGPx in the rat testis. In contrast, cGPx is expressed at low levels, which do not exhibit any unusual cell specificity pattern in the testis.

MATERIAL AND METHODS

PHGPx transcription in isolated whole testes

Testes from early (25 days old) and middle pubertal (60 days old) Wistar rats were decapsulated and incubated at 34°C in RPMI 1640 medium plus glutamine, pyruvate, essential amino acids, and 50 nM sodium selenite in the presence or absence of different agonists: 5 U/ml hCG (Profasi, Serono, Milano, Italy), 10^{-5} M forskolin (ICN Biochemicals, Aurora, Ohio), or 1 μM testosterone. After 4 h of incubation, total mRNA was extracted from testes by a total RNA isolation kit (RNA Fast II, Molecular Systems, San Diego, Calif.). After denaturation with glyoxal and dimethyl sulfoxide at 50°C, 20 μg RNA was separated on a 1.4% agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham, Buckinghamshire, U.K.) (30). Probes (nt 45-733 of pig heart PHGPx cDNA or human β-actin cDNA (Clontech, Palo Alto, Calif.) were labeled with α-^{32}P dATP by a random primer system (Megaprime DNA labeling system, Amersham, Braunschweig, Germany). Hybridizations were performed under standard conditions (30). Results were expressed as ratio of cpm obtained for PHGPx and actin bands after an overnight exposure on Instantimager (Packard, Meriden, Conn.).

Animal treatment

Male Wistar rats were maintained on a standard diet containing 0.38 mg/kg selenium and allowed food and water ad libitum. Ninety-day-old rats received ethane dimethane sulfonate (EDS; prepared according to ref. 31 and dissolved in DMSO/H_2O, 1:3) in a single intraperitoneal injection (75 mg/kg body weight) and were killed at the times indicated. Supplementation of EDS-treated rats with testosterone was performed with a long-acting preparation of testosterone esters (Sustanon, Organon Laboratories, Oss, The Netherlands) injected subcutaneously every 3 days at a dosage of 25 mg/animal. Animal treatments were approved by the 'Comitato di Bioetica' of the Medical Faculty of Padova and met the highest standard for animal care by humans.

Ex vivo enzyme determinations

Testis homogenate was prepared as follows. After decapsulation, testes were weighed and diluted 1:4 w/vol with 50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 5 mM β-mercaptoethanol, and homogenized. After freeze-thawing, the homogenate was centrifuged at 2000 × g for 10 min on a tabletop centrifuge to eliminate unbroken material. The supernatant was assayed for protein content (32) and used for enzyme determinations. For separation of cGPx and PHGPx, 5 ml of the supernatant containing 25 mg/ml protein was applied onto a gel permeation chromatography column (Superdex 75 prep. grade, Pharmacia, Uppsala, Sweden). Fractions of 2.5 ml were collected and assayed for glutathione peroxidase and phenyl esterase activity. Commercially available red blood cell cGPx (Sigma), and purified pig heart PHGPx (33) were used as molecular weight markers in two independent runs.

Glutathione peroxidase activity was measured spectrophotometrically at 340 nm as described (33). Glutathione concentration was 3 mM, final Triton X-100 concentration was adjusted to 0.1%, and the reaction was started with 16 μM PCOOH or 150 μM H_2O_2. To calculate activity, an ε of 6.22 mM^{-1} cm^{-1} was used. The nonspecific NADPH oxidation was subtracted only with the substrate H_2O_2. PCOOH was prepared and titrated as described (33).

Nonspecific phenyl esterase activity was measured spectrophotometrically at 420 nm, following the rate of hydrolysis of 1 mM p-nitrophenyl acetate in 0.1 M Tris-HCl buffer, pH 7.5 (34). As the indicator enzyme for Leydig cells, only phenyl esterase activity eluting in the exclusion volume from Superdex 75 was measured. Aliquots (5–10 μl) of the single fractions from column chromatography were analyzed. Activity rate measurements never exceeded 0.25 OD/min. For calculation of activity, an ε of 18.8 mM^{-1} cm^{-1} was used. The nonspecific hydrolysis of p-nitrophenyl acetate observed in the absence of

[1] Abbreviations: EDS, ethane dimethane sulfonate; cGPx, 'classical' cytosolic glutathione peroxidase (EC 1.11.1.9); PHGPx, phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.12); HE, hematoxylin–eosin; PCOOH, phosphatidylcholine hydroperoxide.

enzyme was subtracted. One unit is the amount of enzyme catalyzing the transformation of 1 μmol min^{-1} at room temperature.

In situ hybridization studies for expression of cGPx and PHGPx

Testes were fixed with paraformaldehyde and serial slices 4 μm in thickness were prepared. For RNA in situ hybridization, a mouse PHGPx-cDNA (180 bp) and a mouse cGPx-cDNA (290 bp) probe cloned into pcDNA3 were used. 'Antisense' and 'sense' RNA probes of PHGPx were generated with *Bgl*II and *Bam*HI linearized vectors using the T7 and SP6 promoters, respectively, and labeled with ^{35}S-UTP to a specific activity of $\geq 10^9$ dpm μg probe. The PHGPx probe corresponded to position 545–738 (Acc. No. D87896). In the case of cGPx, the vector was linearized with *Xba*I and *Bam*HI. The probe comprised position 289–543 and position 760–95, excluding the intron sequence (ref. 35; Acc. No. X03920). The slices were prehybridized at 54°C in a solution containing 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris, 10 mM sodium phosphate pH 6.8, 20 mM dithiothreitol, 0.2× Denhardt's solution, 0.1% Triton X-100, 0.1 mg ml *Escherichia coli* RNA, and 'cold' 0.1 mM α S-UTP. For hybridization, 90,000 dpm μl α^{35}S-UTP labeled RNA probe was added to the hybridization mix and the hybridization was continued at 56°C for 16 h in a humid chamber. The slices were washed in hybridization salt solution with dithiothreitol. After RNase digestion, the slices were washed for 30 min with 2× SSC, 0.1% sodium dodecyl sulfate, 30 min with 0.1% SSC at 37°C, and dehydrated by increasing concentrations of ethanol. The slices were coated with Ilford K5 photoemulsion for autoradiography. After 1–4 wk of exposure at 4°C, the slides were developed in Kodak D19b and slices were Giemsa stained. The sections were analyzed with bright- or dark-field illumination using a Olympus B 201 microscope. Photographs were made using Kodak Ectachrome 160T Professional film. Adjacent slices were stained with hematoxylin–eosin (HE) or processed for macrophage visualization by a monoclonal antibody against murine macrophages (ED2, Serotec, Indianapolis, Ind.). To this end, sections were deparaffinized and incubated in phosphate-buffered saline with 0.3% H_2O_2 for 10 min to destroy endogenous peroxidase activity. Tissue sections were further incubated with 0.1% trypsin (Sigma) for 15 min for antigen retrieval. After treatment with 1% normal horse serum for 10 min, the slices were incubated with the primary antibody at a dilution of 1:400 for 30 min. Treatment with biotinylated anti-mouse immunoglobulin followed, and sections were incubated with peroxidase-conjugated streptavidin for another 30 min. After a final wash, the sections were immersed in a solution containing 0.06 mM 3-3′ diamino-benzidine and 2 mM H_2O_2 in 0.05% Tris-HCl, pH 7.6, for 5 min. Finally, the sections were counterstained with Mayer's hematoxylin. As negative control, normal mouse immunoglobulin G was used instead of the primary antibody.

RESULTS

Steroids and forskolin do not directly affect transcription of the PHGPx gene in testis

To evaluate the hormone responsiveness of the PHGPx gene in its natural context, we incubated decapsulated testes of early and middle puberal rats with 1 μM testosterone, 5 U ml hCG, or 10 μM forskolin and analyzed the PHGPx mRNA level after 4 h of incubation by Northern blotting. Actin mRNA was analyzed for standardization. As seen from **Fig. 1*A*, *B***, the PHGPx mRNA level was found to be lower in early puberal rats than in middle puberal ones, corroborating the previous observation that PHGPx in testis indeed depends on sexual maturation. A roughly fivefold increase in PHGPx mRNA can be estimated between the untreated testes of rats 25 and 60 days old, which compares reasonably with the sixfold increase of PHGPx activity 30 to 60 days after birth reported previously (28). The conventional normalization of the mRNA under investigation by actin mRNA (Fig. 1*C*) led to an underestimation of the increase of PHGPx mRNA, since the actin mRNA in whole testis also increased with sexual maturation.

Incubations with the hormones or forskolin, however, did not significantly affect PHGPx mRNA levels. Thus, a direct transcriptional control of the PHGPx gene by testosterone, hCG, or forskolin, i.e., cAMP, was not detectable by this approach.

The specific activity of PHGPx, not of cGPx, correlates with testicular growth

Left without any evidence corroborating the idea that testosterone, hCG, or cAMP might directly activate PHGPx gene transcription, we decided to reinvestigate the development of PHGPx and cGPx activities of the testis during the prepuberal phase of intact rats. **Figure 2** confirms that the specific activity of PHGPx increases linearly with testis growth during sexual development. In this experiment, GPx activity was measured in unfractionated supernatants with the PHGPx-specific substrate PCOOH and with H_2O_2, which can be reduced by both PHGPx and cGPx. Measurements of both types of activity, when related to testis weight, yielded straight regression lines that differed significantly in slopes and intercepts ($P < 0.05$).

Apparently the main activity may be attributed to PHGPx, since the activities measured with H_2O_2 ranged only slightly above those obtained with the PHGPx-specific substrate. The convergence of the lines further suggests that the relative contribution of cGPx to the total GPx activity shrinks with increased age. To check the accuracy of these assumptions, testis homogenates of early puberal and adult rats were subjected to gel permeation chromatography, taking the difference in molecular mass of the tetrameric cGPx (80 kDa) and the monomeric PHGPx (20 kDa) as an additional criterion of discrimination. As evident from **Fig. 3**, a high and a low molecular mass GPx activity is detected with H_2O_2. With PCOOH, only the low molecular mass GPx activity (i.e., PHGPx) is seen. Comparing the activities of cGPx and PHGPx in early puberal (Fig. 3*A*) and adult rat

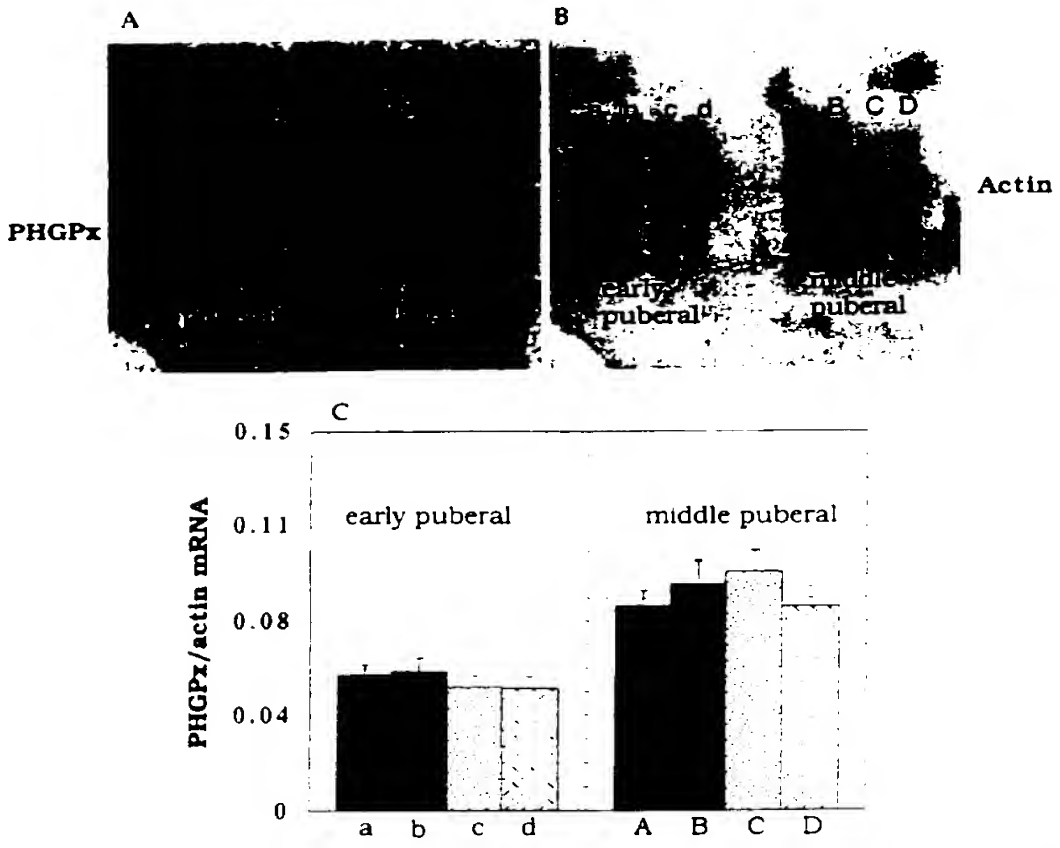

Figure 1. Incapability of testosterone and other agonists to directly induce PHGPx mRNA in testis *in vitro*. Testes from early (25 days old, small letters) and middle puberal animals (60 days old, capital letters) were decapsulated and incubated for 4 h at 34°C in the presence of buffer (a), hCG (5 U/ml) (b), testosterone (1 μM) (c), or forskolin (10^{-5} M) (d). After agarose gel electrophoresis, total mRNA (20 μg) was blotted onto a nylon membrane and probed with radiolabeled PHGPx and actin cDNAs. *A*) A representative blotting of PHGPx mRNA; *B*) the corresponding actin mRNA. *C*) The ratios of cpm of PHGPx and actin mRNA spots are calculated for three independent experiments, as described in Materials and Methods.

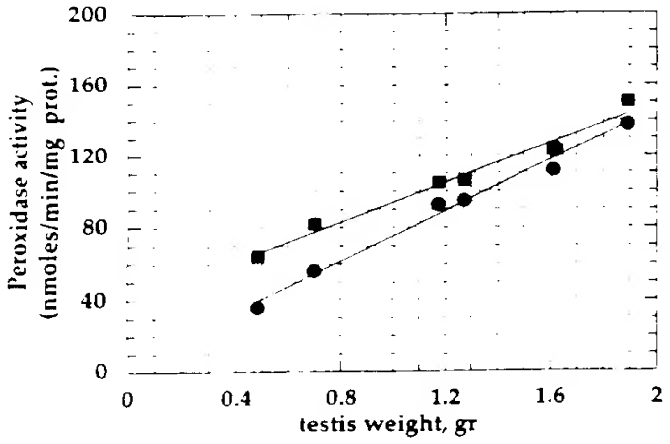

Figure 2. Glutathione peroxidase activity in rat testis as a function of normal testis weight. The glutathione peroxidase activity of rat testis was measured spectrophotometrically with PCOOH (●), or H_2O_2 (■) as substrates directly from homogenates. Results represent the mean of two measurements (one rat each). The two regression lines are significantly different ($P<0.05$). The age of the rats was, from left to right: 32, 40, 47, 57, 54, 64, and 121 days.

testis (Fig. 3*B*), it is evident that PHGPx is increased in the sexually mature testis whereas cGPx remains essentially the same as in early puberal rats. The activity units underestimate the amount of PHGPx roughly by a factor of 10, which implies that PHGPx is already more abundant than cGPx in the testis of young rats and exceedingly high in adults. Thus, the original observation that PHGPx is the main selenoperoxidase in rat testis and correlates with sexual maturation (28) is confirmed, and a relevant change in cGPx is ruled out.

When normally developing rats were treated with hCG (500 IU once a day for 1–2 wk), the rise in PHGPx activity reported for hypophysectomized rats (28) was not observed. In fact, the specific PHGPx activities of hCG-treated rats were consistently lower than that of age- or weight-matched controls (data not shown). This apparent discrepancy in the effects of hCG on testicular PHGPx parallels the differential impact of hCG on the integrity of the seminiferous epithelium. Whereas hCG partially restores the seminiferous epithelium in hypophysectomized rats by

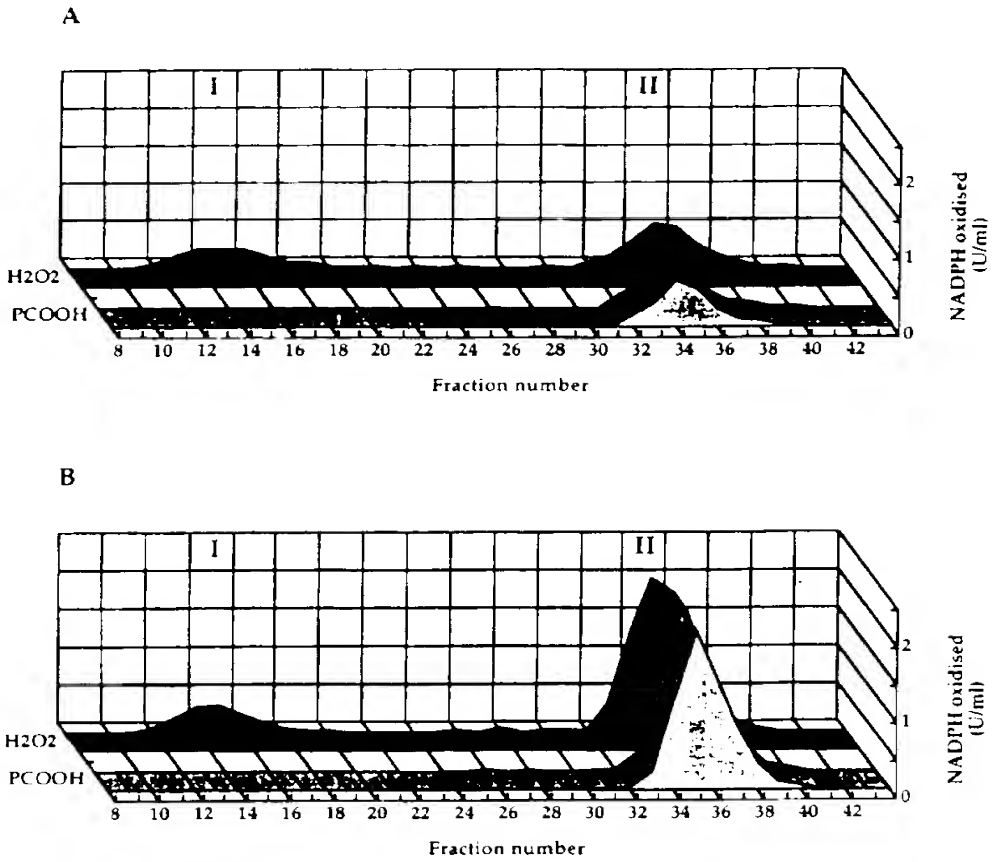

Figure 3. Estimation of cGPx and PHGPx after gel permeation column chromatography of testis homogenate. Rats, 49 (early puberal, testis weight 0.61 g, *A*) or 90 days old (adult, testis weight 1.9 g, *B*), respectively, were used for the experiment. The peroxidase activity was measured with H_2O_2 or PCOOH as substrate. The specific activity measured with H_2O_2 in the fractions 10–20 from the gel permeation column indicates the presence of the tetrameric cGPx (~80 kDa, peak I), whereas the activity observed with H_2O_2 or PCOOH in fractions 30–40 indicates the presence of the monomeric PHGPx (~20 kDa, peak II). With the substrate PCOOH, the total activity applied onto the column was 6.1 U for the 49-day-old rat or 17.3 U for the 90-day-old rat, corresponding to a PHGPx activity recovered after chromatography of 5.5 U (*A*, peak II) and 16.3 U (*B*, peak II). Total glutathione peroxidase activity applied to the columns (measured with H_2O_2 as substrate) was 9.2 and 19.0 U, respectively. Corresponding cGPx activities recovered were 3.4 and 3.6 U, respectively, whereas the remaining units of the samples have to be attributed to PHGPx (peaks II). Integrated PHGPx peaks were essentially the same for both substrates.

substituting for the pituitary stimulation of Leydig cells, it reportedly damages the seminiferous epithelium in normal rats (36, 37).

Testicular PHGPx expression in vivo is mediated by Leydig cell-derived testosterone

The observations reported so far—i.e., lack of evidence for a short-term transcriptional activation of the PHGPx gene, positive correlation of PHGPx activity and mRNA with testicular maturation, and the model-dependent differential effects of hCG on testicular PHGPx—prompted us to reconsider our working hypothesis. Instead of looking for a direct hormonal regulation of PHGPx gene transcription or translation, we turned to the alternative possibility of hormone dependency of a particular cell type that preferentially expresses PHGPx. To evaluate this hypothesis, we treated mature rats with EDS, thereby interrupting the pituitary-gonadal axis. EDS is known to selectively eradicate Leydig cells, which results in a time-delayed atrophy of the seminiferous epithelium and, finally, in sterility (38). In this experimental design, a fast decline of PHGPx activity and/or expression paralleling the loss of Leydig cells would support a direct control of the enzyme by Leydig cell-derived hormones, whereas a delayed response paralleling the atrophy of the seminiferous epithelium would favor the assumption of a prevailing expression in a hormone-dependent cell type.

As shown in **Fig. 4A**, unspecific phenyl esterase, a marker enzyme of Leydig cells, rapidly declines after EDS treatment, reaching negligible levels by day 3 and recovering only marginally by day 28. PHGPx activity also declines upon EDS treatment (Fig. 4A), but

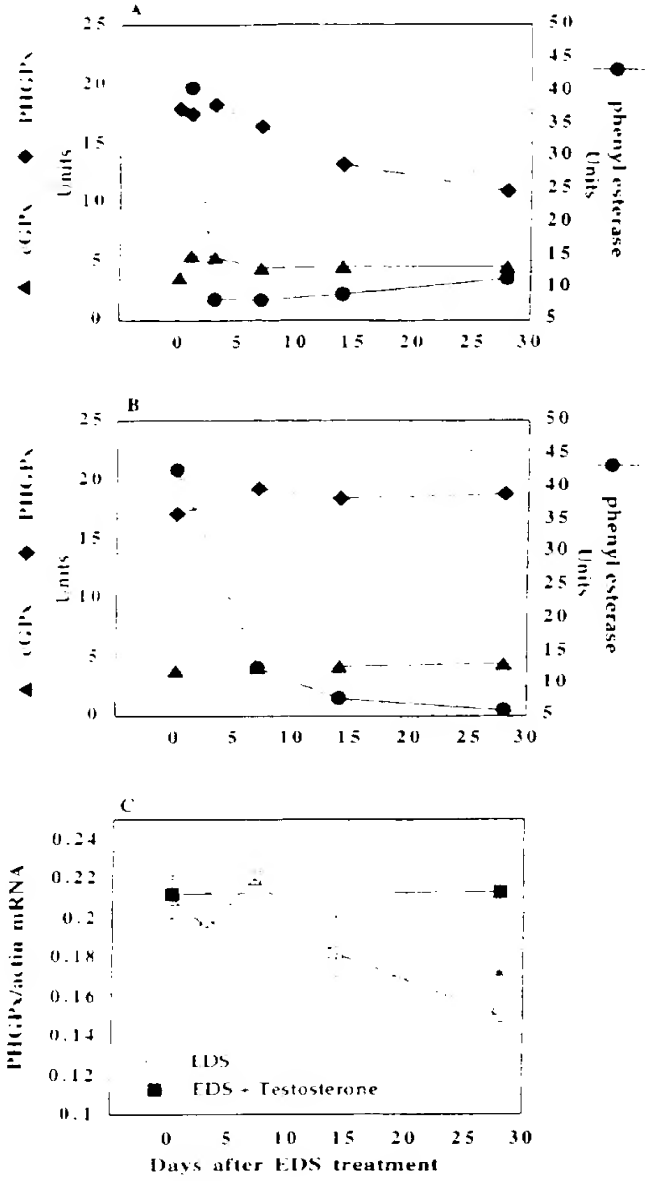

Figure 4. Effect of the treatment with ethane dimethane sulfonate (EDS) without (*A*) or with (*B*) testosterone substitution on phenyl esterase, cGPx, PHGPx activity, or PHGPx mRNA (*C*) of rat testes. Testes were from adult rats (90 days old) receiving a single injection of EDS or a single injection of EDS, followed by testosterone esters (25 mg/animal, every 3 days). *A, B)* Measurements were performed on isolated fractions from gel permeation column chromatography as reported in Materials and Methods and Fig. 3. These were assayed for nonspecific phenyl esterase and glutathione peroxidase activity with p-idrossi phenyl acetate or H_2O_2 as substrate. The peak area, expressed as total units of integrated phenyl esterase (●), cGPx (▲), and PHGPx (◆) activity is reported. *C)* Results obtained by Northern blotting with the same experimental model performed identically three times. The blotting was probed with radiolabeled PHGPx and actin cDNAs and then quantified as described in Fig. 1. $^*P < 0.01$ vs. day 0 and day 28 after EDS plus testosterone administration. Note that PHGPx activity (*A*, ◆) and mRNA (*C*, □) decline much later than phenyl esterase activity (*A*, ●) and that testosterone administration completely prevents the decline of both PHGPx activity (*B*, ◆) and mRNA (*C*, ■).

with considerable delay. Similarly, PHGPx mRNA does not decrease significantly by 4 wk after EDS treatment (Fig. 4*C*). The considerable discrepancy in the time course of Leydig cell eradication and PHGPx activity/mRNA decrease demonstrates that *1)* Leydig cells themselves do not significantly contribute to overall PHGPx content in testis, *2)* testicular PHGPx is obviously not under a direct and short-term control of Leydig cells-derived hormones, but *3)* nevertheless cannot be sustained over time without Leydig cell-derived factors. In view of the data reported above and the known relevance of testosterone to functional integrity of the seminiferous epithelium, we investigated whether the lost Leydig cell function in EDS-treated animals could be substituted by testosterone treatment; indeed, Fig. 4*B, C* shows that, despite a complete and sustained loss of phenyl esterase (or Leydig cells, respectively), the PHGPx activity and mRNA remained completely unchanged for the 4 wk period of observation in the testosterone-substituted animal.

Not unexpectedly, cGPx activities were not altered accordingly by EDS treatment. In fact, they increased shortly after EDS treatment (at the time of ongoing Leydig cell destruction) and returned to control values during the ensuing weeks (Fig. 4*A*). Leydig cells can therefore also be ruled out as a testicular compartment harboring substantial amounts of cGPx.

The round spermatid layer is the site of preferential PHGPx expression

The sites of predominant expression of cGPx and PHGPx were finally clarified by mRNA in situ hybridization. The cGPx mRNA distribution appeared rather diffuse, with a slight predominance in the intertubular interstitium (**Fig. 5*A–D***). Immunostaining of adjacent serial slices with a monoclonal antibody against rat macrophages revealed that interstitial cGPx mRNA was largely associated with macrophages and not with Leydig cells (Fig. 5*E, F*). Accordingly, a similar distribution of cGPx mRNA was observed in testes of rats treated with EDS 28 days earlier and thus largely devoid of Leydig cells (**Fig. 5E**). When hybridized to a probe of comparable size and specific radioactivity, PHGPx mRNA proved to be much more abundant (**Fig. 6**). In the intertubular interstitium, no particular enrichment of PHGPx mRNA in Leydig cells (Fig. 6*E, F*; **Fig. 7*E, F***) or mast cells (Fig. 7*E, F*) was detectable. At best, some association with macrophages was sporadically observed. Instead, a heavily stained inner layer of tubular cells was seen in the majority of tubules (Fig. 6*A–D*). Comparison with hematoxylin eosin-stained slices, in which the cellular morphology is better conserved than in those exposed to the more drastic in situ hybridization conditions, revealed

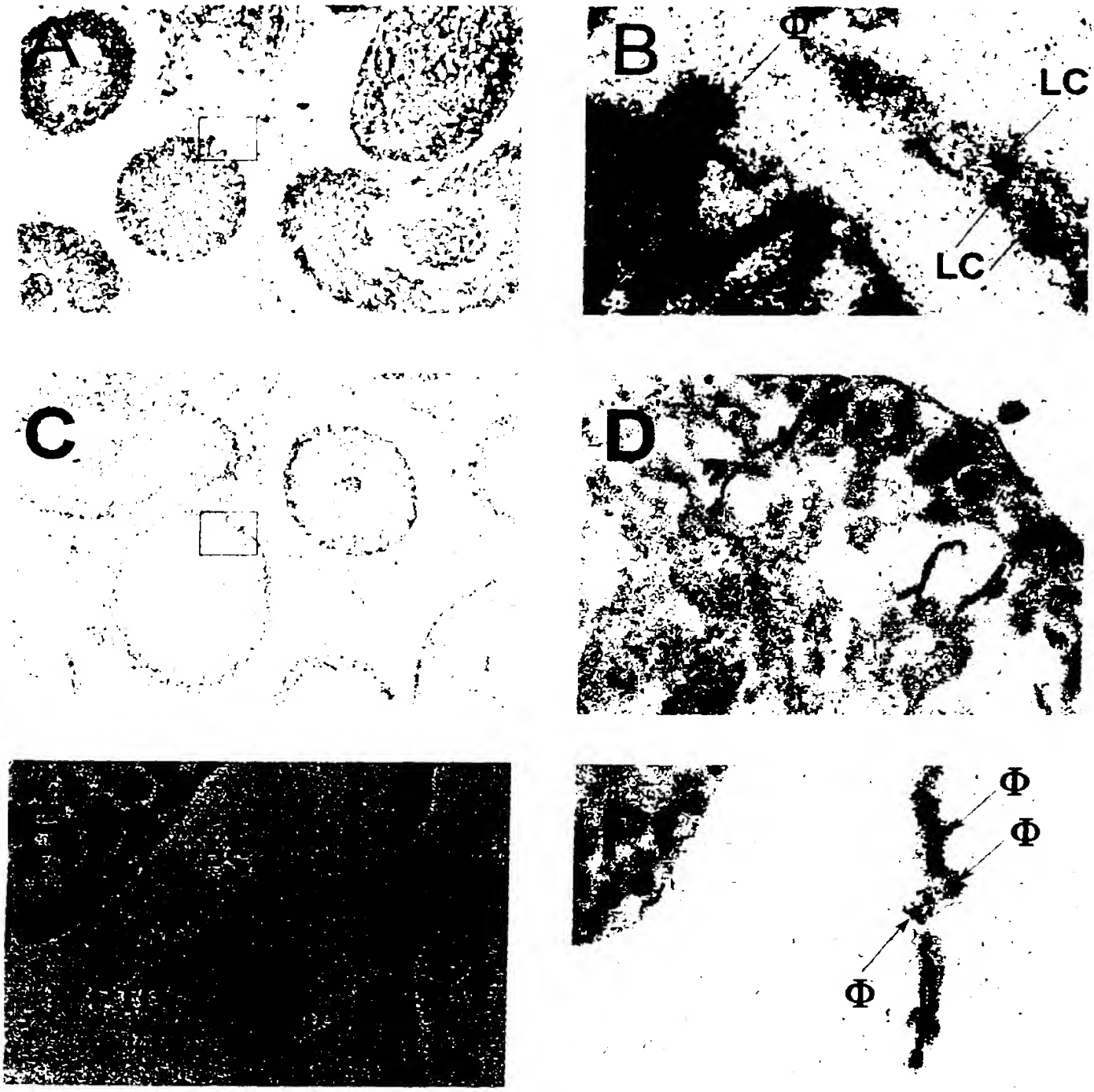

Figure 5 In situ hybridization of cGPx mRNA in rat testis. *A)* In situ hybridization with an antisense probe (see methods) after 4 wk of exposure, magnification 160-fold. *B)* Close-up of panel A, magnification 640-fold; Φ = macrophage, LC = Leydig cell. *C, D)* Sense probe (control) otherwise as in panels *A, B. E)* Intertubular tissue in testis of rats treated with EDS 28 days earlier. Macrophages (deep brown, Φ) are stained by monoclonal antibodies. Note the absence of intact Leydig cells. PS = primary spermatocyte, PTC = peritubular cell, SC = Sertoli cell. *F)* Slice adjacent to E showing in situ hybridization of an antisense probe to cGPx mRNA primarily in macrophages. Exposure time was 2 wk.

that the characteristic label has to be attributed to late round spermatids, whereas the layer containing Sertoli cells, spermatogonia, and primary spermatocytes (but also the elongate spermatids and mature spermatozoa) exhibits a comparable and low background label. This peculiar pattern remains virtually unchanged by EDS treatment (Fig. 7). The small quantitative difference in PHGPx mRNA content

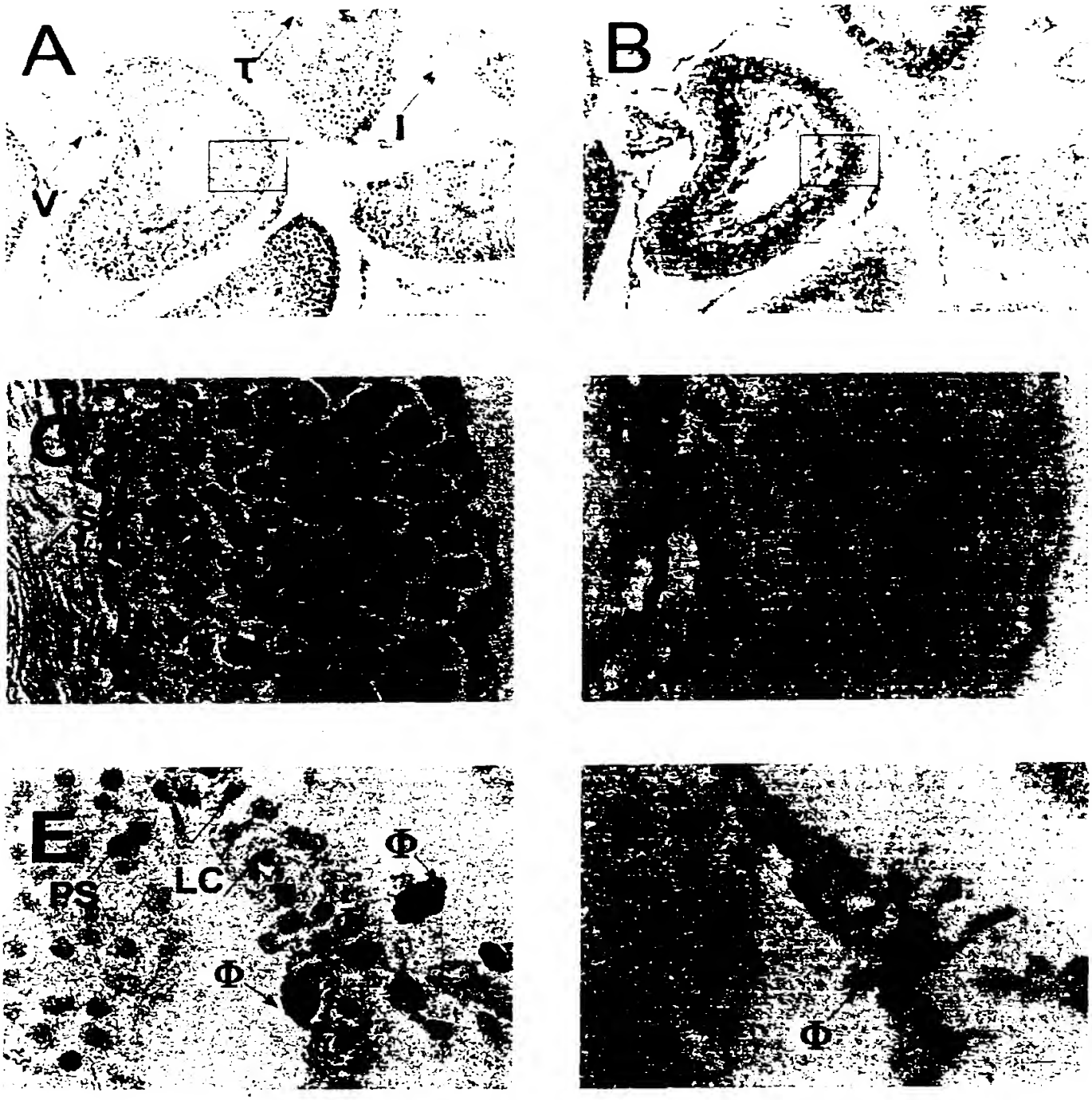

Figure 6. In situ hybridization of PHGPx mRNA in testis of adult normal rats. *A)* HE stain, magnification 160-fold. I = interstitium, T = tubulus, V = blood vessel. *B)* Corresponding in situ hybridization with an antisense probe (see Materials and Methods) after 1 wk of exposure and staining with Giemsa. The slice adjacent to *A* was used. *C)* Close-up of panel *A*, magnification 640-fold; ES = elongated spermatid, RS = round spermatid, SZ = spermatozoa. *D)* Close-up of panel *B*, magnification 640-fold. *E)* Intertubular tissue stained for macrophages (deep brown, Φ), magnification 640-fold. *F)* Section adjacent to panel *E* showing PHGPx mRNA.

found by transcript quantitation in whole testis of normal and EDS-treated rats (see Fig. 4*C*) could not be verified histologically due to the various stages of the spermatogenic cycle presented in the tubules of a given slice. In fact, the only obvious changes observed histologically in the EDS-exposed testes were decrease of elongate spermatids and mature spermatozoa, lack of Leydig cells, and enrichment with mast cells (Fig. 5*E*, 7*E*), which is in agreement with earlier findings (38).

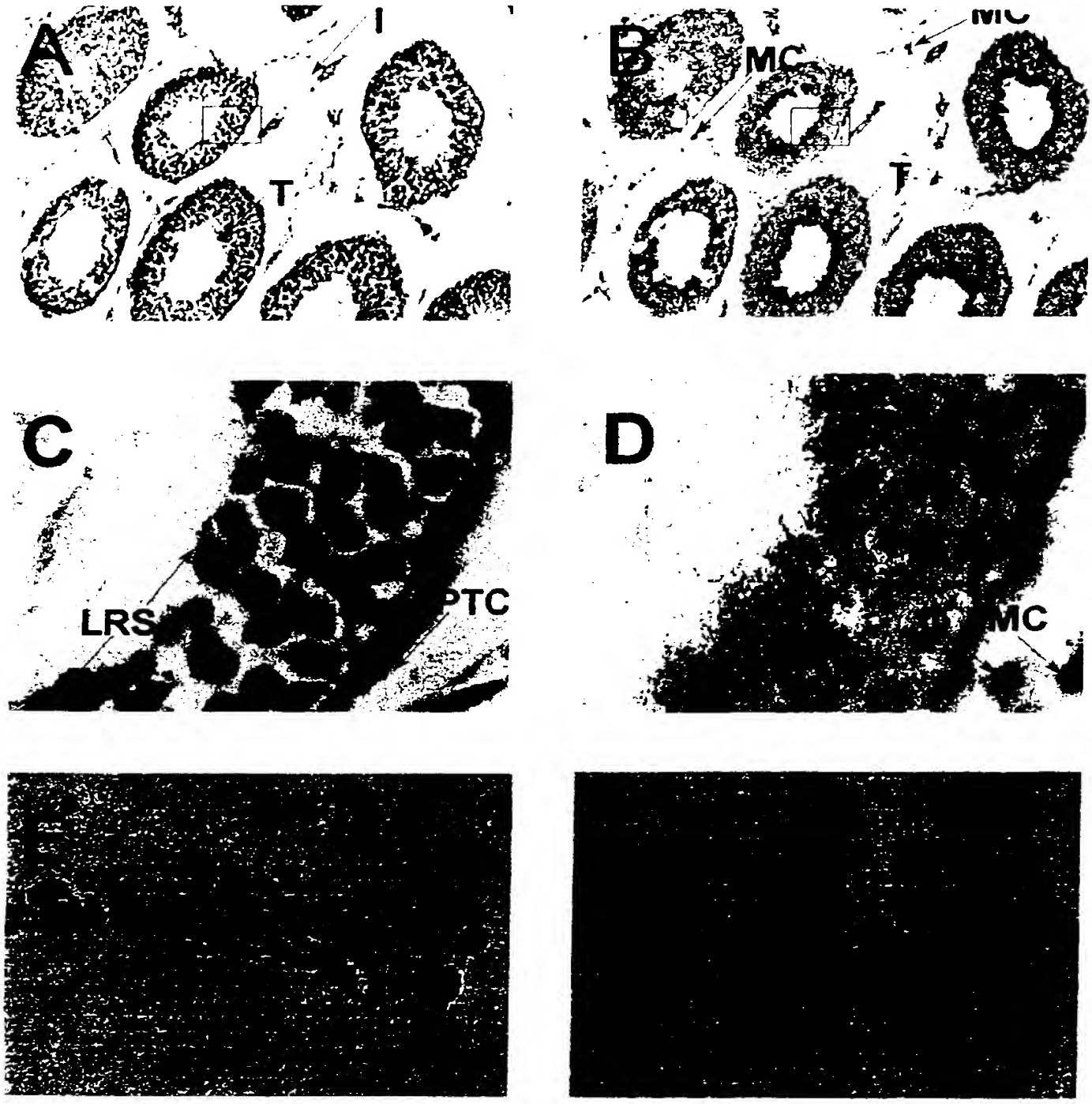

Figure 7. In situ hybridization of PHGPx mRNA in testis of rats treated with EDS 28 days earlier. Note the lack of mature spermatozoa, almost complete disappearance of Leydig cells and the infiltration of mast cells. *A)* HE stain; magnification 160-fold. *B)* Corresponding in situ hybridization with an antisense probe (see methods) after 1 wk of exposure and staining with Giemsa. The slice adjacent to *A* was used. MC = mast cell. *C)* Close-up of panel *A*; magnification 640-fold; LRS = late round spermatids. *D)* Close-up of panel *B*; magnification 640-fold. *E)* Intertubular tissue stained for macrophages (deep brown, Φ), magnification 640-fold. *F)* Section adjacent to *E* shows PHGPx mRNA.

DISCUSSION

The data presented here do not suggest any specific role for cGPx in testicular function. It is uncharacteristically expressed in testis with the exception of a slight predominance in interstitial macrophages. Also, its activity and expression is not altered during sexual maturation or by hormonal treatment. The

transient increase of cGPx observed during Leydig cell destruction by EDS is most likely explained by an increased infiltration of macrophages reportedly occurring precisely within this time frame (38). The phenomenon thus has to be regarded as a consequence of tissue remodeling after chemical Leydig cell destruction and appears to be unrelated to any hormonal disturbance resulting therefrom.

The irrelevance of cGPx to specific testicular function may also be inferred from recent gene disruption experiments. cGPx-negative mice developed normally and were obviously fertile even in the homozygous state (39). Only their susceptibility to oxidative stress, as demonstrated by paraquat exposure, was dramatically increased (40). These observations classify cGPx as an emergency device to balance unspecific oxidative stress. This role of cGPx may be considered important for the prevention of hydroperoxide-mediated mutagenic events also in the germ line, as it probably is in somatic cells.

In contrast, PHGPx appears to be intimately involved in the process of spermatogenesis. Admittedly, an essential role of PHGPx in spermatogenesis awaits final proof, since specific PHGPx inhibitors are not available, genetic deficiencies have not yet been discovered, and PHGPx knock-out animals have not been constructed. But the peculiar expression pattern of PHGPx in the seminiferous epithelium as well as its dependency on testosterone is intriguing. Preferential PHGPx gene transcription is observed in the late round spermatids, which ceases abruptly upon transition to elongated spermatids. It therefore is tempting to speculate about a specific role of PHGPx in the prefinal stage of spermatogenesis comprising the change in shape from round spermatids to the elongated forms. We have no idea how PHGPx may trigger this process of differentiation. We can, however, safely state that this differentiation process itself depends on testosterone and that PHGPx expression exhibits persuasive parallelism. Both phenomena occur only after puberty; both are prevented by hypophysectomy, are decreased by pharmacological Leydig cell destruction, and are fully restored by administration of testosterone alone in Leydig cell-deprived testis. It may also be inferred from the data presented here that the sudden transcription of the PHGPx gene at a particular phase of spermatogenesis does not likely result from a direct transcriptional control by testosterone, but rather is due to a testosterone-dependent factor selectively expressed in the spermatids, that still awaits identification.

The regulatory role of PHGPx we infer here is not as remote as it may appear at first glance. PHGPx is indeed an unusual representative of the GPx family of proteins, and is not likely to be an enzyme responsible simply for the defense against oxidative stress. Although it is homologous to and shares the catalytic center with its congeners (21), it has been questioned whether PHGPx is adequately classified as a *glutathione* peroxidase, because all residues implicated in the specific binding of glutathione by cGPx are missing and the rate of the reaction with GSH is comparatively low (14). It therefore remains uncertain whether PHGPx uses the major cellular reductant GSH efficiently enough to constitute a defense system against oxidative damage. The tissue distribution of PHGPx, being low in the lung, liver, and kidney but high in testis (41, 42), does not parallel any obvious demand for antioxidants. Similarly, the only nonvertebrate homologue of PHGPx discovered so far is almost exclusively associated with the female gender of *Schistosoma mansoni* and with the vitelline glands there, which are indispensable for reproduction (43, 44). A role of PHGPx distinct from defense against peroxide damage is further suggested by the lack of correlation of protein levels determined immunologically and pertinent glutathione peroxidase activities (45). Considerable protein levels with low or absent enzymatic activity are detectable, for example, in liver and spermatozoa (28), respectively, and this PHGPx protein appears to be closely associated with other proteins yet to be identified (F. Ursini, unpublished observation). A putative regulatory role of PHGPx has also been discussed due to its variable subcellular distribution (14) and its unique ability to reduce hydroperoxides of complex biomembrane lipids generated by 15-lipoxygenase (46), an enzyme implicated in reticulocyte differentiation processes (47). Clear evidence for the involvement of PHGPx in cellular responses has been reported in two cases: *1)* PHGPx levels manipulated by time-controlled selenium deprivation and resupplementation correlated inversely with endotoxin-triggered leukotriene biosynthesis *in vivo* (48); and *2)* overexpression of PHGPx in a human endothelial cell line abrogated the interleukin 1-dependent NFκB activation (49). Whether such regulatory events are achieved by modulating the cellular peroxide tone is still debatable. It may as well be envisaged that the selenium moiety of PHGPx is oxidized by particular hydroperoxides and then reacts with susceptible thiols of proteins subject to redox regulation.

The evident potential of PHGPx to regulate responses in various cellular systems strengthens the idea that its peculiar expression pattern in testis is somehow related to spermatid differentiation. If so, its selenoprotein nature might explain the male infertility observed in selenium deficiency. However, beyond the two selenoperoxidases discussed so far, other links of selenium biochemistry with testicular function have to be considered. Certainly, a decline in thioredoxin reductase recently shown to contain a selenocysteine residue (18) and to respond to selenium deprivation in tissue culture (23) would inevitably lead to an impairment of the rapidly dividing seminiferous epithelium due to disturbance of the

thioredoxin-dependent deoxynucleotide metabolism (50). Thioredoxin reductase activity, however, is less easily affected by selenium deprivation (23) than the glutathione peroxidases under consideration (25). Still, selenoprotein P, a protein containing 10–12 selenocysteine residues of unknown function, is expressed in Leydig cells (51); pulse-labeling experiments with ^{75}Se showed an additional selenoprotein of 34 kDa to be expressed in rat testis after onset of puberty (52), as is PHGPx (28). In view of this emerging complexity, we anticipate other selenoproteins to complement PHGPx in the testicular maturation process.

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Method to detect male antifertility problems

The invention relates to a method to detect male antifertility problems based on the determination of latent phospholipid hydroperoxide glutathione peroxidase (PHGPx).

Selenium is essential for male fertility. In mature mammalian spermatozoa it is largely restricted to the midpiece harbouring the helix of mitochondria embedded into a keratine-like selenium-enriched matrix called the mitochondrial capsule. Selenium deficiency is associated with impaired sperm motility, structural alterations of the midpiece up to breakages, and loss of *flagellum*. The predominant selenoprotein of the mammalian male reproductive system, phospholipid hydroperoxide glutathione peroxidase (PHGPx), was shown to be preferentially expressed in round spermatids but was hardly detectable in terms of messenger RNA or activity in spermatozoa. The basis of the invention is the discovery that PHGPx persists in spermatozoa but as insoluble, enzymatically inactive material forming the mitochondrial capsule. PHGPx activity of this material can be restored by high concentrations of thiols. PHGPx, thus, acts as a peroxidase in the proliferating

germ epithelium to prevent oxidative damage. In the late stages of sperm maturation it is oxidatively cross-linked to become a structural element indispensible for sperm function. Accordingly, the determination of the PHGPx content in sperm or any other tissue of humans or life stock can be used to estimate the fertilization potential of sperm.

The invention thus in accordance with claim 1 provides a method for the determination of latent phospholipid hydroperoxide glutathione peroxidase (PHGPx) comprising the steps of

a) obtaining a sperm sample,

b) solubilizing the spermatozoa by using detergents and chaotropic agents and reactivating latent PHGPx by using high concentrations of thiols and

c) determining enzymatic activity of reactivated latent PHGPx.

In a further aspect the invention relates to the use of the inventive method in a method for predicting the fertilizing potential of spermatozoa in sperm samples.

Further advantageous and/or preferred embodiments of the invention are subject-matter of the subclaims.

In a preferred embodiment of the inventive method an additional step of removing any reactivating reagents by, e.g., gel filtration is provided between the step of solubilizing the spermatozoa and the step of determining the enzymatic activity of reactivated latent PHGPx.

In a further embodiment of the invention instead of determining enzymatic activity of reactivated latent PHGPx the con-

tent of solubilized PHGPx is determined by conventional immunological techniques or measurement of enzymatic activity.

The used chaotropic agent is, for example, 4 - 8 M guanidine chloride, 4 - 8 M guanidine thiocyanate or 5 - 8 M urea.

The used thiol is, for example, 50 - 300 mM 2-mercaptoethanol, 25 - 300 mM dithiothreitol (DTT) or dithioerythritol (DTE).

The sperm sample is, for example, from humans or life stock.

In the following the invention is disclosed in more detail with reference to examples and to drawings. However, the described specific forms or preferred embodiments are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the following description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Regarding the cited literature a reference list with more detailed bibliographic information can be found at the end of this specification.

Routine preparations of rat sperm mitochondrial capsules (1) yielded a fraction which was insoluble in 1% SDS and 0.2 mM DTT and displayed expected vesicular appearance in electron microscopy (Fig. 1 a). The vesicles readily disintegrated upon exposure to 0.1 M mercaptoethanol (Fig. 1 b) and became fully soluble in 6 M guanidine-HCL. When the solubilized cap-

sule material was subjected to gel electrophoresis essentially four bands in the 20 kDa region were detected (Fig. 1 c, left lane). Western blotting revealed that the most prominent one reacted with antibodies directed against PHGPx (Fig. 1 c, right lane) which is undetectable as active peroxidase in mature spermatozoa (Tab. 1). Also, N-terminal sequencing of the 21 kDa band representing about 46% of total protein content according to Coomassie stain revealed that it consisted of at least 95% pure PHGPx. Puzzled by this unexpected finding, we investigated the composition of the mitochondrial capsules in more detail by 2D-electrophoresis (Fig. 2 a) followed by microsequencing and/or MALDI-TOF for identification (Fig. 2 b). For this purpose the capsules were dissolved completely in a buffer designed for electrophoretic separation of membrane proteins (see Methods). The spot migrating with an apparent molecular weight of about 21 kDa and focussing at a pH near 8 (spot 3) proved to be PHGPx according to the masses of tryptic peptides detected by MALDI-TOF (Fig. 2 b). By the same technique, also the slightly more acidic charge isomer (spot 4), the more basic ones (spots 1, 2 and 5) as well as the spots 6 and 7 exhibiting a smaller apparent molecular mass were shown to contain PHGPx (Fig. 2 c). The predicted N-terminal (pos. 3-12) and C-terminal peptides (pos. 165-170), the fragment corresponding to positions 100-105 and those expected from the basic sequence part 119-151 were too small to be reliably identified. Interestingly, the fragment corresponding to positions 34-48 comprising the active site selenocysteine was not detected either. With these exceptions, however, the MALDI-TOF spectra unequivocally complied with the PHGPx sequence and thus proved the presence of PHGPx in spots 1-7. On a thicker 2D-gel developed with a non-linear gradient from pH 3-10 also five distinct spots were detected

in the 20 kDa region. In this experiments the presence of PHGPx was verified by microsequencing of major tryptic peptides (not shown). Again the spots representing PHGPx were the most prominent ones present in the gel.

The spots 1-6 of Fig. 2 a proved to be essentially homogeneous. As is exemplified in Fig. 2 b, the fragments yielding MALDI-TOF signals of significant intensities could be attributed to PHGPx. Only in the minor spot 7 a trace of impurity was detected, which was tentatively identified as a subunit of the T cell receptor variable region (acc. no. 228109). Based on integrated stain intensities of the individual spots those representing PHGPx amounted to about 50% of the capsule material. Most of the minor components (see Fig. 2 a) are not likely constituents of the capsule, which is believed to be built up by apposition of extramitochondrial proteins onto the outer mitochondrial membrane. In other gels further proteins like the mitochondrial glutathione S-transferase subunit Yb-2 (acc. no. 121719) and an endothelin converting enzyme (acc. no. 1706564) could be identified by MALDI-TOF or micro-sequencing (not shown). Spots 8 and 9 were identified as the "outer dense fiber protein", a cystine-rich structural sperm protein, which is associated with the helix of mitochondria in the sperm midpiece but also extends into the *flagellum* (7). In view of the nature of the additional proteins detected, the PHGPx content of the actual mitochondrial capsule should substantially exceed the 50% observed by gel scanning.

Despite intense search, we could not detect any trace of the "sperm mitochondria-associated cysteine-rich protein ("SMCP") (7) in our capsule preparation. This cysteine- and pro-

line-rich protein had for long been considered the selenoprotein accounting for the selenium content of the mitochondrial capsule in sperm (1,8,9). Cloning of the rat SMCP gene, however, revealed that it did not contain any in-frame TGA codon enabling selenocysteine incorporation (10). In mice, the three in-frame TGA codons proved to be upstream of the translation start (7). In developing mouse sperm SMCP stayed cytosolic up to states in which the mitochondrial capsule was already formed and only became superficially associated with the outer mitochondrial membranes of late spermatids and epididymal spermatozoa (7). SMCP thus is not necessarily an integral part of the mitochondrial capsule nor it is a selenoprotein. Instead, the "mitochondrial capsule selenoprotein (MCS)", as SMCP was originally referred to (1,7-10), is indeed PHGPx.

The chemical modifications of PHGPx leading to distinct differences in charge and apparent MW could not be reliably elucidated. Sequencing revealed an identical N-terminus of the size isomers starting with ASRDDWRCAR, i.e. a sequence either corresponging to the originally proposed translation start (11) after cleavage of the first two residues or derived from a possible pre-PHGPx (12) after processing of a mitochondrial leader peptide (13). Tryptic fragments extending towards the C-terminus up to position 164 were consistently observed also with the faster migrating specimen (Fig. 2 c) which leaves little room to explain an apparent MW difference of 1 to 1.5 kDa. As to the charge isomers, it may be recalled that a potential phophorylation had been inferred from early attempts to sequence pig heart PHGPx (14). The assignment of masses to possibly phosphorylated tryptic peptides, however, remained equivocal. Certainly, more trivial events such as

deaminations of Gln and Asn residues, C-terminal degradation, oxidation of the active site selenium, or its elimination might have contributed to the charge heterogeneity.

PHGPx as the major component of the sperm mitochondrial capsule had so far escaped attention, since as such it is enzymatically inactive, as it generally is in mature spermatozoa prepared from the tail of the epididymis (Tab. 1). It is neither reactivated by glutathione in the low millimolar range as used under conventional test conditions. High concentrations of thiols (0.1 M 2-mercaptoethanol or dithiothreitol), which in the presence of guanidine fully dissolve the capsule, regenerate a significant PHGPx activity, as measured after elimination of denaturating and reducing agents (Tab. 1). In fact, the specific activities thus obtained from mitochondrial capsules exceed, by a factor of 20, the highest values ever measured, i.e. in spermatogenic cells. Nevertheless, this extreme PHGPx activity is still low compared to its content in PHGPx protein. Based on the specific activity of pure PHGPx, the reactivated enzyme would be equivalent to less than 3% of the capsule protein, whereas the 2D-electrophoresis suggests a PHGPx protein content of at least 50%. It is worth noting that the same reductive procedure does not increase the specific activity of PHGPx in spermatogenic cells from testicular tubules (Tab. 1). The switch of PHGPx from a soluble active enzyme to an enzymatically inactive structural protein thus occurs during final differentiation of spermatozoa.

The alternate roles of PHGPx, being either a glutathione-dependent hydroperoxide reductase or a structural protein, are not necessarily unrelated. One of the features common to

all glutathione peroxidases is a selenocysteine residue which together with a tryptophan and a glutamine residue forms a catalytic triad (15,16). Therein the selenol group of the selenocysteine residue is dissociated and highly activated by hydrogen bonding to reduce hydroperoxides with high rate constants. The reaction product, a selenenic acid derivative, R-SeOH, will readily react with thiols, e.g. GSH, to form an intermediate with a selenadisulfide bridge between enzyme and substrate, R-Se-S-G, from which the ground state enzyme can be regenerated by a second GSH. PHGPx is unique among the glutathione peroxidases in several respects: i) It usually is monomeric having its active site freely accessible at the surface; this facilitates interaction with bulky substrates. ii) Arginine residues surrounding the active site and specifically binding glutathione in most types of glutathione peroxidases are lacking in PHGPx (16); correspondingly, its specificity for the reducing substrate is less pronounced (16). It therefore can be envisaged that oxidized PHGPx may form diselenide or selenadisulfide bridges with exposed SeH or SH groups of proteins (16) including PHGPx itself, and this process, possibly followed by SH/SS, SH/SeS, or SH/SeSe exchange reactions, will create cross-linked protein aggregates. This ability of PHGPx might become particularly important if cells are exposed to hydroperoxides at extremely low concentration of glutathione, as is documented for late states of spermatogenesis (17-20). Fig. 3 is to mimick the oxidative events occurring during sperm maturation. Short term exposure of soluble proteins derived from spermatogenic cells to moderate H_2O_2 concentrations in the absence of GSH yields a variety of PHGPx-containing high molecular weight aggregates. Undoubtedly, therefore, PHGPx, by means of its intrinsic enzymatic potential, can catalyse oxidative protein

aggregation using protein thiols as alternate substrates. During sperm maturation, PHGPx thereby transforms itself into an enzymatically inactivated structural protein. This view, however, is not to imply that PHGPx could not depend on additional proteins when building up the highly organized architecture of the spermatozoal midpiece.

Our findings require a fundamental reconsideration of the role of selenium in male fertilty. The intriguing predominance of the selenoprotein PHGPx in the male reproductive system has so far been believed to reflect the necessity to shield germ line cells from oxidative damage by hydroperoxides and reactive oxygen species derived therefrom (11,17,21,22). This concept still merits attention with regard to the mutagenic potential of hydroperoxides and probably holds true for the early phases of spermatogenesis where PHGPx is still present as an active peroxidase (6,21). At this stage related activities reported for PHGPx or other glutathione peroxidases, e.g. silencing lipoxygenases (23), dampening the activation of NF B (24) or inhibiting apoptosis (25), may also be relevant. In later stages of spermatogenesis characterized by a shift of the redox status resulting in loss of GSH (18-20,26), the ability of PHGPx to use protein thiols as alternate substrates opens up new perspectives of redox regulation which remain to be explored. In the mature spermatozoon PHGPx has experienced a pronounced metamorphosis now being a major constituent of the keratinuous material embedding the mitochondrial helix. It appears revealing that precisely this architectural pecularity in the midpiece of spermatozoa shows gross structural alterations in selenium deficiency. We therefore assume that the mechanical instability of the midpiece observed in selenium deficiency is a con-

sequence of an impaired PHGPx biosynthesis. This view implies that it is not the antioxidant capacity of PHGPx which is crucial for male fertility but its ability to utilize hydroperoxides to build an indispensable structural element of the spermatozoon.

Any shortage of PHGPx during sperm maturation, be it due to selenium deficiency, other reasons of inhibited biosynthesis or inhibition of activity should therefore result in disturbed sperm midpiece architecture and, in consequence, loss of fertilization potential of sperm. This conclusion was further corroborated by determination of reactivated PHGPx in sperm of individuals with documented fertility problems. The latter were divided into three groups: depending on whether i) intrauterine sperm injection (iui) or ii) conventional in-vitro-fertilisation (ivt-et) was still successful or iii) intracytoplasmatic sperm injection was required (icsi). As shown in Fig 4, the PHGPx values differed markedly between these groups. While the iui group displayed values close to normal, PHGPx in the icsi group was almost absent, the ivf-et group ranking in between. The reasons of the diverse PHGPx content being unknown, the data reveal that markedly reduced PHGPx content in sperm is incompatible with normal male fertility. Similarly, there is a strong correlation between "typical" sperm appearance (Fig 5) and "fast" moving sperm with PHGPx content (Fig 6). This correlation, however, shows marked scattering of data indicating that PHGPx content of sperm is not the only reason of abnormal shape and motillity of sperm. It should also be pointed out that the sperm samples were taken from individuals without any obvious disease suggesting that extremely reduced PHGPx levels are well tolerated.

Taken together, the ovservation that PHGPx builds up an esssential structure of sperm and that its content in sperm correlates with the fertilization potential leads to the inventive concept to use the PHGPx content of sperm as a predictive parameter for the necessary measures to overcome male fertility problems. To this end, it appears necessary to reactivate the PHGPx contained in sperm in order to estimate its content by either immunological methods or by any of the established determinations of its specific activity. (28, 29).

Methods

Preparation of rat spermatozoa, tubular cells and mitochondrial capsule

Spermatozoa of four month old Wistar rats (about 300 grams of body weight) were collected by squeezing *cauda epididymis* and *vas deferens* in phosphate buffer saline (PBS) and by centrifugating at 600 x g for 10 minutes. Cell and sperm pellets were layered on a discontinous 45%, 70% and 95% Percoll gradient and centrifugated at 300 x g for 20 min. Spermatogenic cells stacked on top of the gradient, while spermatozoa separated into the 70% Percoll layer. Cells from seminiferous epithelium were prepared as follows (26): testes were deprived of *albuginea*, seminiferous tubules were cut into small pieces in PBS containing 0.250 mg/ml collagenase, and incubated twice 25 °C for 15 min. Cells then were filtered through a stainless steel screen (140 μm pore), washed in PBS and centrifugated at 300 x g for 10 min. Sperm mitochondrial capsule was prepared according to Calvin et al.(1): sperms were resuspended in 0.05 M Tris - HCl pH 8.0 at the concen-

tration of 10^6 cells/ml and treated with trypsin (0.2 mg/ml) for 10 minutes. After stopping the protease action with trypsin inhibitor (0.5 mg/ml) and SDS (10 mg/ml) sperms were centrifugated at 1,500 x g for 10 minutes. Pellets were resuspended in 0.05 M Tris - HCl, pH 8.5 containing 1% sodium dodecyl sulphate (SDS), and 0.2 mM DTT and kept under continuous stirring for 30 minutes. Following centrifugation at 4,500 x g for 15 min, the resulting supernatant was layered on a 1.6 M sucrose cushion. After centrifugation for 20 min at 18,000 x g in a swinging rotor, sperm capsules were collected as a band at the top of the sucrose cushion, washed in Tris - HCl, pH 8.0 and spun at 140,000 x g.

1D-electrophoresis and Western blotting

Electrophoresis was performed according to Laemmli under either reducing (+ 2- mercaptoethanol) or non-reducing conditions. Proteins were blotted onto nitrocellulose, probed with an antigen-purified rabbit antibody raised against pig heart PHGPx and detected by biotinylated anti rabbit IgG and streptoavidin alkaline phosphatase complex.

2D-electrophoresis

100μg of the mitochondrial capsule material was dissolved in 400 μl of a solution containing of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 20 mM Tris base and 0.5% IPG buffer (Pharmacia) and focused in an IPG-phor (Pharmacia) at 20°C by stepwise increasing voltage up to 5000 V but not exceeding a current of 30 μA per IPG strip. The pH gradient was non-linear from 3-10 or linear from 3-10 or 6-11. The focussed IPG strips were then equilibrated for SDS electrophoresis (10 min each) with a solution containing 60 mM DTT in 6 M urea, 30% glycerol, 0.05 M Tris-HCl buffer pH 8.8 and in the same

buffer where DTT was substituted by 250 mM iodoacetamide. After SDS-electrophoresis (12% polyacrylamide) the gels were stained with Coomassie.

Protein identification

Coomassie-stained spots were cut out from the gels, neutralized with $(NH_4)HCO_3$, destained with 400 µl 50% acetonitrile/10 mM $(NH_4)HCO_3$ and dried in a Speed Vac Concentrator. Protein digestion was done overnight using 2 ng/µl sequencing grade trypsin (Promega) in 50 mM $(NH_4)HCO_3$ (Boehringer, Mannheim). The resulting peptides were extracted twice with 60% acetonitrile / 40% H_2O / 0.1% TFA. Extracts were combined and lyophilized in the Speed Vac Concentrator. Peptide digests were desalted on small RP18-columns, eluted with saturated α-hydroxy-4-cyano-cinnamic acid and loaded directly onto the MALDI target (27). Reflectron MALDI mass spectra were recorded on a Reflex™ MALDI/TOF-mass spectrometer (Bruker-Franzen-Analytik, Bremen). The ions were excellerated at 20 kV and reflected at 21.3 kV. Spectra were externally calibrated using the monoisotopic MH^+ ion from two peptide standards. 100-200 laser shots were summed up for a single mass spectrum. Mass identifcation was performed with MS-Fit (http://falcon.ludwig.ucl.ac.uk/ucsfhtml/msfit.htm).

Alternatively, protein spots from 1.5 mm 2D-gels were digested with modified trypsin (Promega, sequencing grade) in 25 mM $(NH_4)HCO_3$ overnight at 37°C. The digests were extracted twice and dried as before and reconstituted in 10µl water. Peptides were separated on a reversed-phase capillary column (0.5 mm x 150 mm) with a gradient of acetonitrile in 0.1% formic acid / 4 mM ammonium acetate at a flow rate of 5µl/min and collected manually. Aliquots of 5 µl were spotted onto

Biobrene-treated glass fiber filters and sequenced on an Applied Biosystems 494A sequencer with standard pulsed-liquid cycles. Before N-terminal sequencing, proteins were blotted from polyacrylamide gels onto PVDF membranes for 16 h at pH 8.3 (25 mM Tris-HCl, 192 mM glycine) and 100 mA (30 V).

When applicable, PHGPx was also identified by activity measurement according to (28) using the specific substrate phosphatidylcholine hydroperoxide.

Figure 1 Presence of PHGPx in the mitochondrial capsule of spermatozoa.
a, Mitochondrial capsule prepared by trypsination and centrifugation according to (1) at 80,000 fold magnification. **b**, The same preparation as shown in **a**, but after exposure to 0.1 M 2-mercaptoethanol for 15 min at 4°C. Contamination of the capsule material by mitochondria is evident from the presence of mitochondrial ghosts. **c**, SDS gel electrophoresis of proteins extracted from capsule material (see Methods) by treatment with 0.1 M 2-mercaptoethanol, 0.1 M Tris-HCl, pH 7.5, and 8 M guanidine HCl. Left lane is stained with Coomassie, right lane demonstrates presence of PHGPx by Western blotting.

Figure 2 showws the analysis of the composition of the mitochondrial capsule of spermatozoa
a, 2D-electrophoresis of purified dissolved capsule material. Proteins were focused in a linear pH-gradient from 3 to 10 (horizontal direction), then reduced, amidocarboxymethylated, subjected to SDS-electrophoresis, and stained with Coomassie. MALDI-TOF analysis of the visible spots identified the following proteins (SwissProt data base): spot 1-7 PHGPx (MW 19

443; pI 8.27; acc. no. 544434); spots 8 and 9, outer dense fiber protein (MW 27351; pI 8.36; acc. no. P21769); spots 10 and 11, voltage-dependent anion channel-like protein (MW 31720; pI 7.44; acc. no. 540011); spot 12, "stress-activated protein kinase" (MW 48107; pI 5.65; acc. no. 493207); spot 13, glycerol-3-phosphate dehydrogenase (MW 76479; pI 5.86; acc. no. P35571).

b, MALDI-TOF spectrum (overview) of tryptic peptides obtained from PHGPx as found in spot 3. Abscissa, mass/charge ratio of the peptide fragments; ordinate, arbitrary units of intensity; numbers at mass signals, identified peptides in the PHGPx sequence (see insert for position numbers); T, trypsin-derived fragments.

c, Compilation of tryptic PHGPx fragments identified in spots 1-7 by MALDI-TOF. Vertical lines designate potential tryptic cleavage sites. Dark blocks, identified typical cleavage products; shadowed blocks, masses resulting from incomplete cleavage or equivocally assignable to different fragments (e.g. 3-9 and 63-69).

Figure 3 shows the formation of PHGPx-containing aggregates from spermatogenetic cells by H_2O_2 in the absence of GSH. Spermatogenic cells were homogenised in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ml pepstatin A, 0.7 µg/ml leupeptin and 5mM 2-mercaptoethanol at pH 7.5 and 4°C. After centrifugation at 105,000 x g for 30 min, excess reagents were removed by gel permeation using NAP 5 columns equilibrated with 10mM Tris-HCl, 0.15 M NaCl, 1mM EDTA and 0.1% Triton X-100, pH 7.5. Immediately (t 0) and 15 min after (t 15) the addition of 75 µM H_2O_2 aliquots of the mixture (0.05 mg of protein) were withdrawn and subjected to electrophoresis under (a) re-

ducing and (b) non reducing conditions. After blotting on nitrocellulose, PHGPx was detected by specific antibodies.

Figure 4 shows the PHGPx specific activity in extracts (0.1% Triton X-100 and 0.1 M 2-mercaptoethanol of human sperm. Correlation between this parameter and therapeutic appproach in cases of couple infertility.

Figure 5 shows the relationship between PHGPx specific activity and number of "typical" sperms per milliliter of semen. "Typical" is a morphological parameter of sperm evaluation.

Figure 6 shows the relationship between PHGPx specific activity and number of "fast" sperms per milliliter of semen. "Fast" is a parameter of sperm mobility.

Table 1 PHGPx activity in spermatogenic cells, spermatozoa and sperm capsule. Effect of thiols.

Preparation	mU/mg protein [a, b]
Cells from seminiferous tubules	
5 mM 2-mercaptoethanol [c]	250 ± 10
100 mM 2-mercaptoethanol [c]	260 ± 10
Spermatozoa from tail of epididymis	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	3,140 ± 200
Mitochondrial capsule	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	5,600 ± 290

[a] One enzyme mU catalyzes the reduction of one nanomole of phosphatidylcholine hydroperoxide per minute at 37 °C in the presence of 3 mM GSH.

[b] Data are the mean and S. D. of five independent measurements.

[c] Solubilisation / reduction was carried out in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ ml pepstatin A, 0.7 µg/ml leupeptin and 2-mercaptoethanol as indicated at pH 7.5 and 4 °C for 10 min Low molecular weight compounds were removed before activity determination by a NAP 5 cartridge.

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Claims

1. Method for the determination of latent phospholipid hydroperoxide glutathione peroxidase (PHGPx) comprising the steps of
   a) obtaining a sperm sample,
   b) solubilizing the spermatozoa by using detergents and chaotropic agents and reactivating latent PHGPx by using high concentrations of thiols and
   c) determining enzymatic activity of reactivated latent PHGPx.

2. Method according to claim 1, wherein between said step of solubilizing the spermatozoa and said step of determining the enzymatic activity of reactivated latent PHGPx an additional step of removing any reactivating reagents is provided.

3. Method according to claim 2, wherein said additional step of removing any reactivating reagents is performed by gel filtration.

4. Method according to any of the preceeding claims, wherein instead of determining enzymatic activity of reactivated latent PHGPx the content of solubilized PHGPx is determined by conventional immunological techniques or measurement of enzymatic activity.

5. Method according to any of the preceeding claims, wherein the chaotropic agent is 4 - 8 M guanidine chloride, 4 - 8 M guanidine thiocyanate or 5 - 8 M urea.

6. Method according to any of the preceeding claims, wherein the thiol is 50 - 300 mM 2-mercaptoethanol, 25 - 300 mM dithiothreitol (DTT) or dithioerythritol (DTE).

7. Method according to any of the preceeding claims, wherein the sperm sample is from humans or life stock.

8. Use of a method of any of the preceeding claims in a method for predicting the fertilizing potential of spermatozoa in sperm samples.

FIG.1

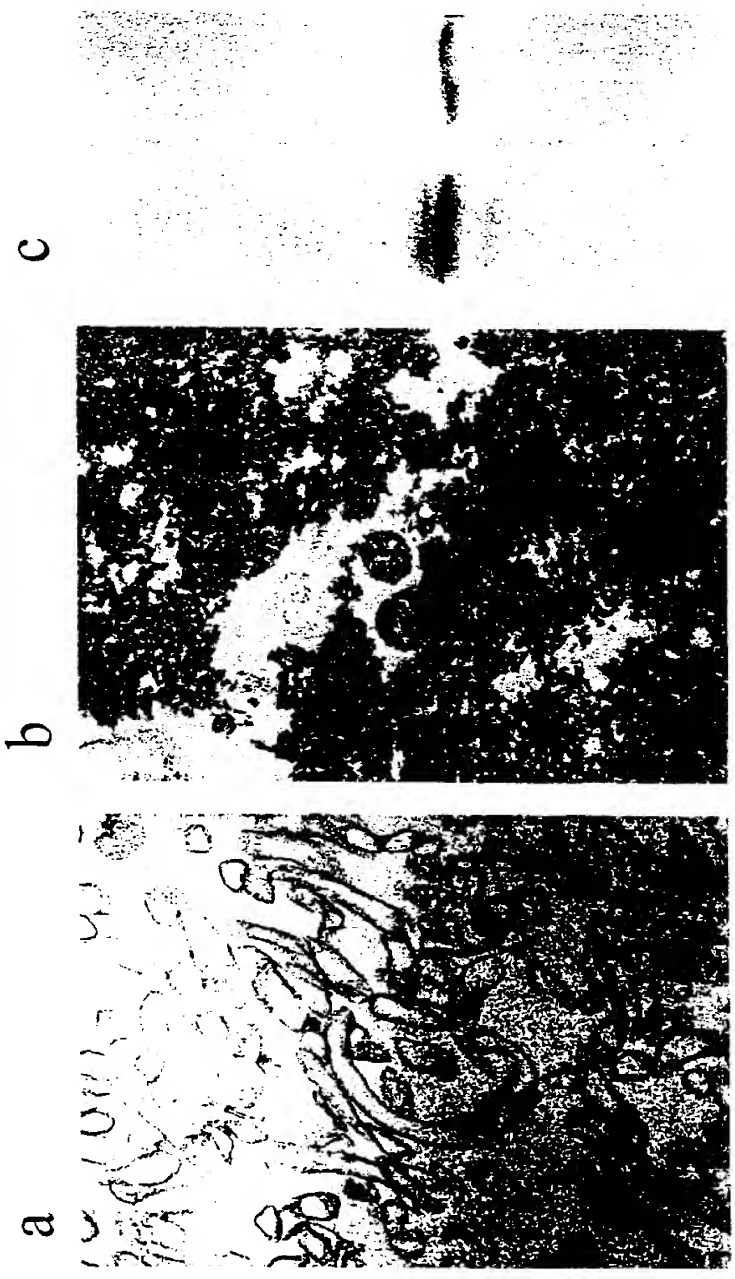
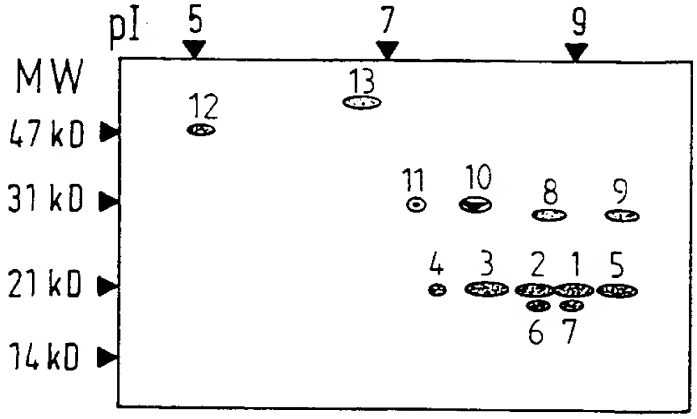
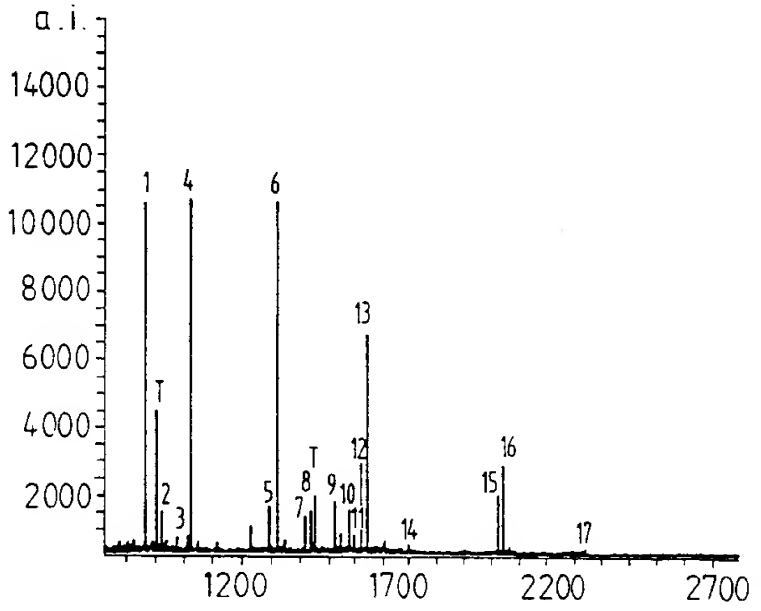


Fig. 2

a)



b)



1= 63 - 69
2= 13 - 20
3= 6 - 12
4= 91 - 99
5= 141 - 151
6= 70 - 80
7= 153 - 164
8= 153 - 164 (Met-ox)
9= 106 - 118
10= 152 - 164
11= 152 - 164 (Met-ox)
12= 21 - 33
13= 49 - 62
14= 91 - 105
15= 81 - 99 (pyro-Glu)
16= 81 - 99
17= 70 - 90

c)

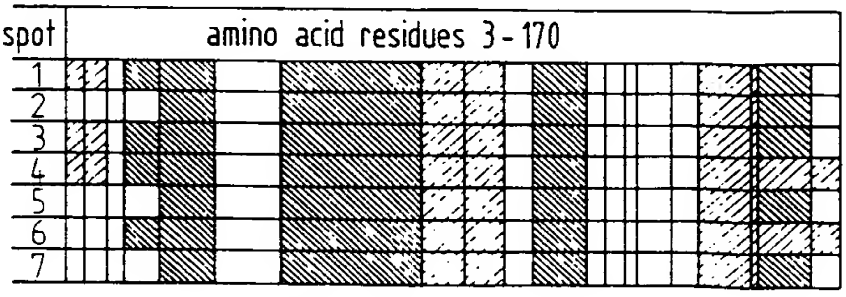


Fig. 3

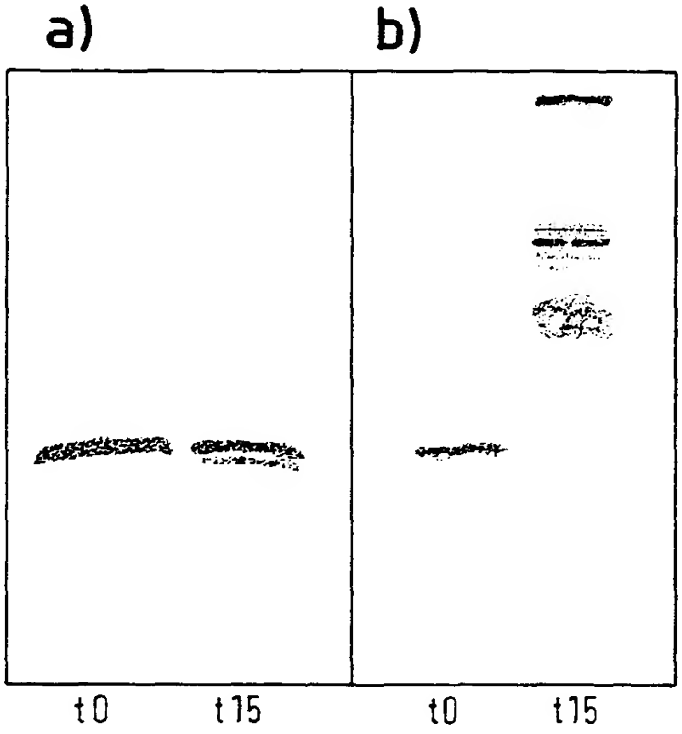

a)
b)
t0
t15
t0
t15

Fig. 4

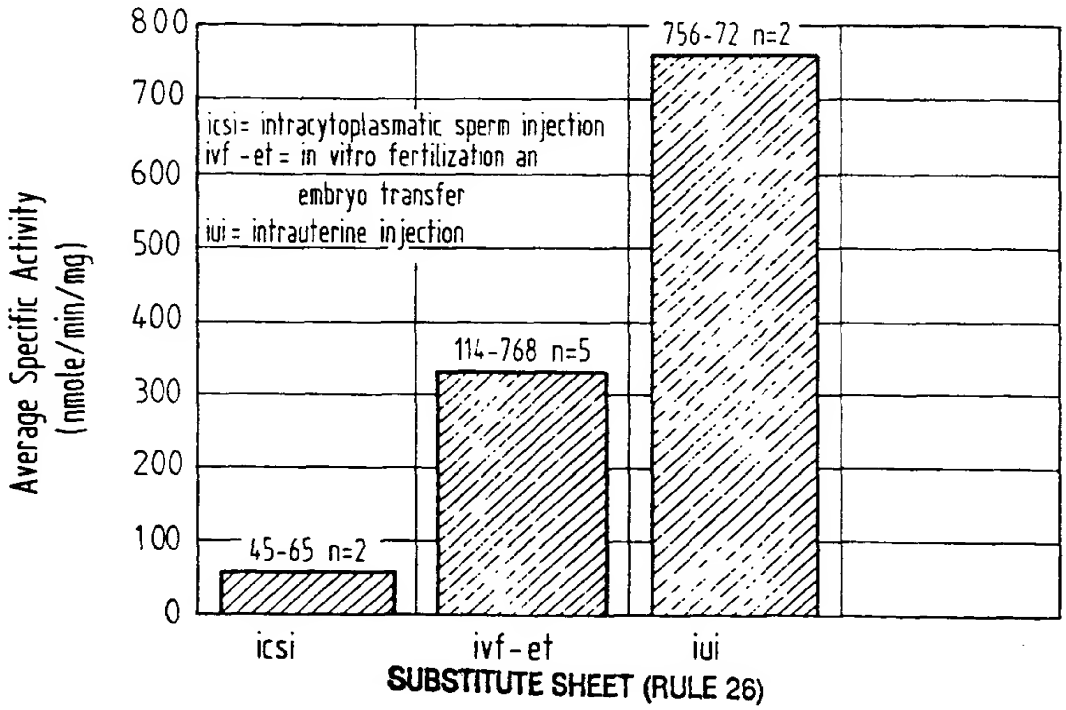

800
700
600
500
400
300
200
100
0
Average Specific Activity
(nmole/min/mg)
icsi= intracytoplasmatic sperm injection
ivf -et = in vitro fertilization an
embryo transfer
iui = intrauterine injection
45-65 n=2
114-768 n=5
756-72 n=2
icsi
ivf-et
iui

Fig. 5

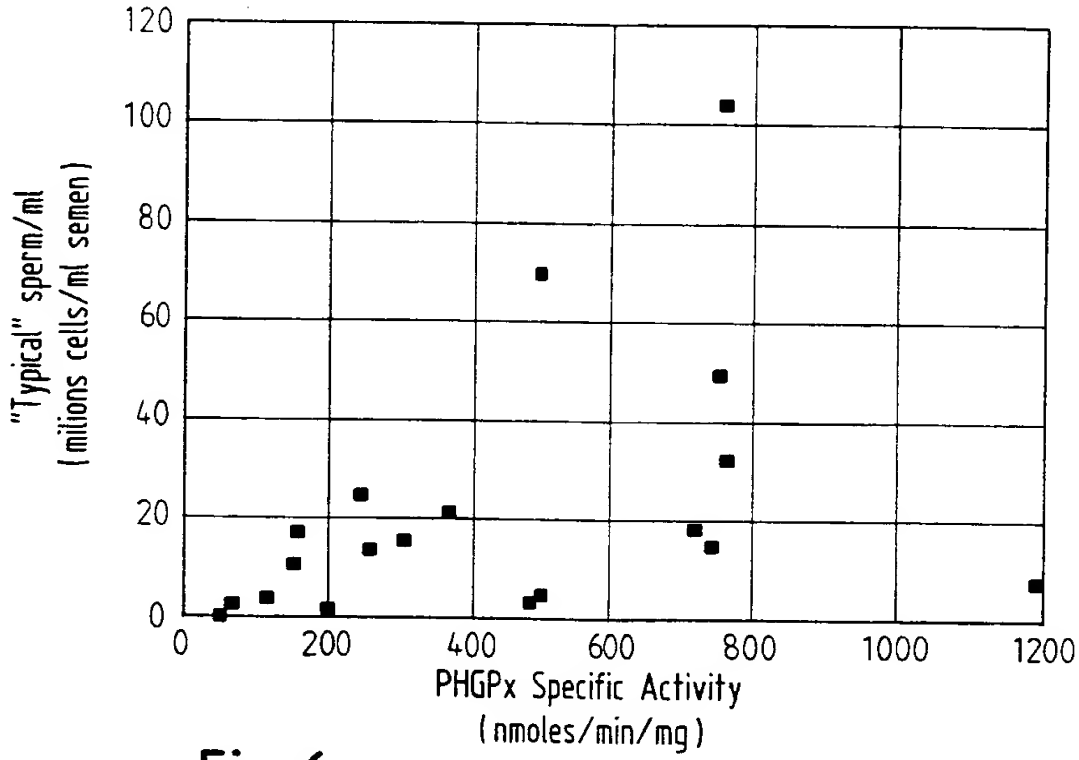
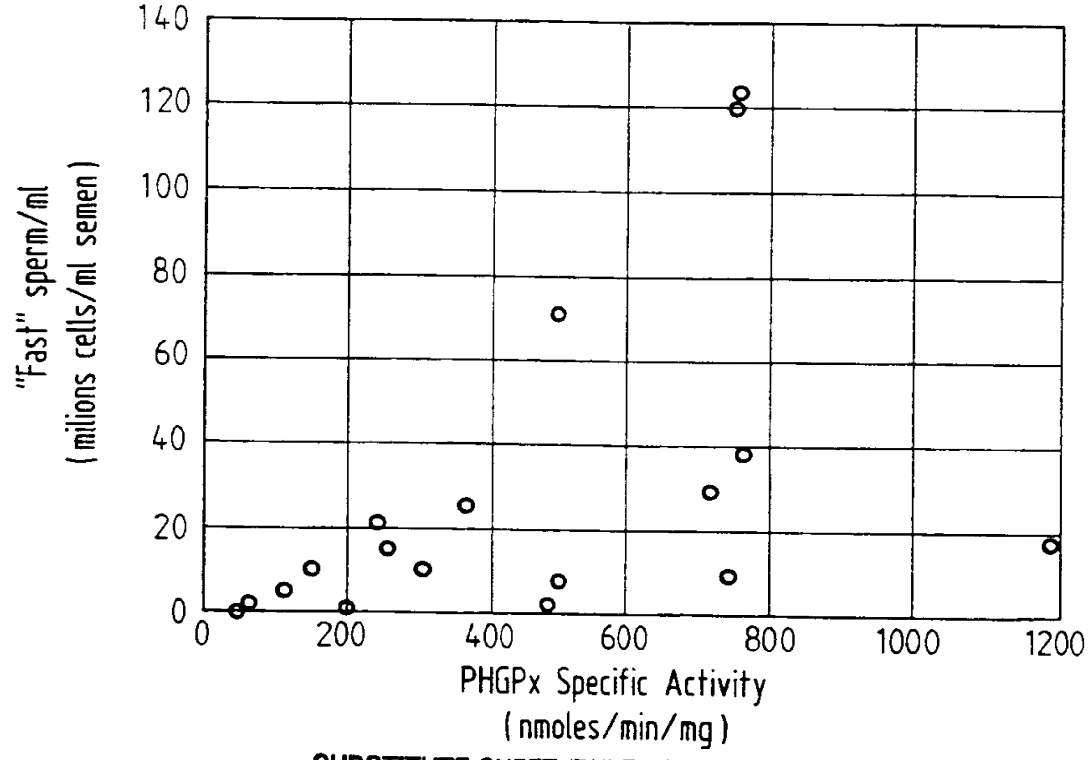

"Typical" sperm/ml
(milions cells/ml semen)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)

Fig. 6


"Fast" sperm/ml
(milions cells/ml semen)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/01877

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/573 G01N33/561 C12Q1/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROVERI A. ET AL.: "Enzymatic and immunological measurements of soluble and membrane bound PHGPx" METHODS ENZYMOL., vol. 233, 1994, pages 202-212, XP000921475 cited in the application page 204, paragraph 5 -page 210, paragraph 1 ---	1-8
X	MAIORINO M. ET AL.: "Phospholipid hydroperoxide glutathione peroxidase" METHODS ENZYMOL., vol. 186, 1990, pages 448-457, XP000921458 page 452, paragraph 3 -page 455, paragraph 5 --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2000	25/07/2000

Name and mailing address of the ISA	Authorized officer
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3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 13225 A (BETH ISRAEL HOSPITAL) 9 May 1996 (1996-05-09) claims 12-19	1-8
X	MAIORINO M. ET AL.: "Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation" FASEB J., vol. 12, 1998, pages 1359-1370, XP002141807 page 1360, column 2, paragraph 4 page 1361, column 2, paragraph 3 -page 1363, column 1, paragraph 1 page 1368, column 1, line 21-27	1-8
P,X	URSINI F. ET AL.: "Dual function of the selenoprotein PHGPx during sperm maturation" SCIENCE, vol. 285, 27 August 1999 (1999-08-27), pages 1393-1396, XP002141939 page 1394, column 3, paragraph 3 -page 1395, column 1, paragraph 1	1-8

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/01877

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613225 A	09-05-1996	US 5895749 A	20-04-1999
		AU 4018295 A	23-05-1996
		CA 2203828 A	09-05-1996
		EP 0789538 A	20-08-1997
		JP 11514204 T	07-12-1999
		US 5935800 A	10-08-1999